Univerzita Karlova v Praze

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Katedra farmakologie a toxikologie

Interakce antiretrovirotik s lékovými efluxními transportéry a jejich vliv na transplacentární farmakokinetiku

Interactions of antiretrovirals with drug efflux transporters and their role in the transplacental pharmacokinetics

Dizertační práce

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Mgr. Zuzana Ptáčková

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Abstrakt

Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

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Název dizertační práce: Interakce antiretrovirotik s lékovými efluxními transportéry a jejich vliv na transplacentární farmakokinetiku

Prevence přenosu viru HIV z matky na dítě je založená na podávání kombinované antiretrovirální léčby a to ideálně po dobu celého těhotenství. Jedním z mechanismů profylaktického působení antiretrovirotik je jejich přítomnost ve fetální cirkulaci, což však může být zároveň spojeno s nepříznivými účinky na vyvíjející se plod. Pro optimalizaci léčebné strategie i minimalizaci rizik spojených s léčbou HIV pozitivních těhotných žen je důležitá podrobná znalost všech faktorů ovlivňujících přestup antiretrovirotik z matky k plodu přes placentární bariéru.

Náplní této práce bylo zjistit, zda v transplacentární farmakokinetice vybraných léčiv hrají úlohu lékové efluxní transportéry, o kterých je známo, že svou činností chrání plod před působením cizorodých látek. S pomocí použitých *in vitro, in vivo, in situ* a *ex vivo* metodik jsme zhodnotili míru vlivu lékových efluxních transportérů na distribuci vybraných léčiv mezi matkou a plodem.

U léčiv zidovudinu, abakaviru a tenofoviru disoproxil fumarátu jsme potvrdili a v některých případech jako první popsali, že vykazují chování substrátů placentárních ABCB1 a ABCG2 transportérů. V případě abakaviru a zidovudinu však pasivní difúze a/nebo účast jiných transportérů napomáhá k distribuci těchto léčiv do plodu. Naproti tomu transplacentární přestup léčiva lamivudinu ani parentní látky tenofoviru není činností ABC efluxních transportérů ovlivněn. Dále jsme zjistili, že dlouhodobá

aplikace tenofoviru a emtricitabinu březím potkanům nevede k ovlivnění exprese hlavních ABC transportérů ve vybraných orgánech plodu ani matky.

Prezentované výsledky přispívají k celkovému obrazu problematiky transplacentárního přestupu antiretrovirotik. Mají svůj význam při sestavování terapie HIV pozitivních žen, hodnocení faktorů ovlivňujících bezpečnost a účinnost profylaxe léčebných režimů a poukazují na možnosti lékových interakcí jednotlivých antiretrovirotik.

Abstract

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Title of doctoral thesis: Interactions of antiretrovirals with drug efflux transporters and their role in the transplacental pharmacokinetics

The combination antiretroviral therapy that should be administered during the whole pregnancy is the backbone of prevention of mother-to-child transmission of HIV infection. One of the prophylactic mechanisms of such treatment is the presence of antiretrovirals in the fetal circulation. However this can be associated with the potentially harmful effects of drugs on the developing fetus. To select optimal therapy while minimizing risks it is inevitable to have detailed knowledge of all the factors affecting transplacental transport of drugs.

The aim of this study was to detect whether drug efflux transporters are able to protect fetus against xenobiotics can affect the transplacental pharmacokinetics of the selected antiretroviral drugs. Employing variety of *in vitro*, *in vivo*, *in situ* and *ex vivo* methods we determined the role of the drug efflux transporters in the distribution of drugs between mother and fetus.

We suggested that antiretrovirals zidovudine, abacavir and tenofovir disoproxil fumarate to be the substrates of placental ABCB1 and ABCG2 transporters. However, passive diffusion and/or other transporters enabled the penetration of abacavir and zidovudine into the fetus. Conversely, the transplacental transport of lamivudine and parent drug tenofovir was not affected by the activity of ABC efflux transporters. Further we detected that long-term administration of tenofovir and emtricitabine to

pregnant rats altered expression of the main drug efflux transporters in the selected organs of neither fetus nor mother.

The presented results contribute to the complex knowledge regarding transplacental pharmacokinetics of antiretroviral drugs. These findings should be taken into account when antiretroviral therapy of HIV positive pregnant women is selected and the risk factors and prophylactic efficacy of the particular regimens are assessed. Moreover our results indicate the potential of drug-drug interactions of antiretrovirals tested.

Obsah

1 Seznam zkratek

ABC	„ATP-binding cassette“ rodina transportérů
ABCB1	P-glykoprotein, MDR1
ABCC2	Multidrug Resistance-Associated Protein 2, MRP2
ABCC5	Multidrug Resistance-Associated Protein 5, MRP5
ABCG2	Breast Cancer Resistance Protein, BCRP
AZT	zidovudin
cART	kombinovaná antiretrovirální terapie, z angl. combination antiretroviral therapy
EMA	Evropská léková agentura, z angl. European Medicines Agency
ENT	ekvilibrativní nukleosidový transportér, z ang. equilibrative nucleoside transporter
FDA	Úřad pro kontrolu potravin a léčiv, z angl. Food and Drug Administration
F/M poměr	poměr mezi plazmatickými hladinami léčiva v pupečníkové a mateřské krvi
MATE	Multidrug and Toxin Extrusion Protein
MVM vezikuly	vezikuly z izolované mikrovilózní membrány, z angl. microvillous plasma membrane
MTCT	přenos z matky na dítě, z angl. mother-to-child transmission
NIH	Národní institut zdraví, z angl. National Institute of Health
NNRTI	ne-nukleosidový inhibitor reverzní transkriptázy, z angl. non-nucleoside reverse transcriptase inhibitor
NRTI	nukleosidový/nukleotidový inhibitor reverzní transkriptázy, z angl. nucleoside/nucleotide reverse transcriptase inhibitor
OAT	transportér pro přenos organických aniontů, z angl. organic anion transporter
OATP	organické anionty transportující polypeptidy, z angl organic anion-transporting polypeptides
OCT	transportér pro přenos organických kationtů, z angl. organic cation transporter
PI	inhibitor proteázy, z angl. protease inhibitor
SLC	„Solute Carrier“ rodina transportérů

TFV	tenofovir
TDF	tenofovir disoproxil fumarát

2 Úvod

Epidemie infekce HIV zůstává závažným celospolečenským problémem. Každoročně se tímto virem nakazí více než dva miliony lidí, z nichž významnou část tvoří ženy v reprodukčním věku [1]. Odhadem až 1,5 milionu HIV pozitivních žen každý rok otěhotní a přivede na svět dítě, které by bez lékařského zásahu mělo téměř padesátiprocentní šanci, že se od své matky tímto virem nakazí [2]. Díky zlepšující se dostupnosti zdravotní péče se podařilo v loňském roce snížit počet narozených dětí s HIV na 220 tisíc, což představuje významný pokrok ve srovnání se situací na počátku tisíciletí, kdy tento počet přesahoval půl milionu [1].

Zásadní přelom v péči o těhotné HIV pozitivní ženy představuje zavedení farmakologické antiretrovirální léčby do praxe v druhé polovině devadesátých let minulého století. Na počátku této terapeutické intervence stály výsledky první klinické studie prokazující že podání zidovudinu (AZT) těhotným ženám a jejich novorozencům snižuje riziko přenosu HIV o téměř 70 % [3]. Následovala řada studií (z nichž mnohé stále probíhají), které srovnávají účinek a bezpečnost užívání antiretrovirotik těhotnými ženami [4]. V současnosti je na trhu k dispozici řada těchto léčiv ať už samostatně nebo ve fixních kombinacích, které se používají u těhotných žen za účelem léčby jejich nemoci a zároveň ke snížení rizika přenosu viru z matky na dítě (MTCT, z angl. mother-to-child transmission).

Hlavní strategií Světové zdravotnické organizace v péči o těhotné HIV pozitivní ženy je snaha více zpřístupnit optimální léčbu ženám tak, aby se v budoucnu rodilo co nejméně nakažených dětí [5]. Protože nejdůležitějším krokem v rámci prevence MTCT je podání léčiv infikovaným matkám a jejich ohroženým dětem [6], je nezbytné vybírat kombinační režimy tak aby byly účinné a zároveň pro oba bezpečně.

3 Teoretická část

3.1 Mechanismy přenosu HIV z matky na dítě

Přítomnost viru HIV byla zjištěna ve tkáních plodů z potratů z prvního i druhého trimestru těhotenství, které byly provedeny u infikovaných žen [7, 8]. Protilátky proti HIV byly nalezeny ve fetální i placentární tkáni osmitýdenních plodů [9], což spolu s předchozími studiemi potvrzuje schopnost transplacentárního přestupu viru během časných fází těhotenství. Za kritické období z hlediska přenosu HIV je považován porod, kdy je dítě vystaveno přímému působení viru v tělních tekutinách matky a vir se pak může dostat do oběhu novorozence [10]. Protože HIV přechází do mateřského mléka, je dále možná i nákaza během kojení [10]. Ačkoliv je obtížně přesně stanovit, kdy došlo k přenosu viru (např. negativní testy na přítomnost HIV v krvi novorozence těsně po porodu nevylučují, že k přenosu viru již došlo *in utero*), je všeobecně přijímán fakt, že s největší pravděpodobností dochází k nákaze během porodu (45 - 50 % případů), méně pak v průběhu těhotenství (15 – 20 %) a během kojení (30 – 40 %) [11]. Při péči o HIV pozitivní těhotné ženy tak musí být bráno na zřetel riziko přenosu infekce v jakékoliv fázi těhotenství a po porodu.

3.2 Mechanismy antiretrovirální profylaxe přenosu HIV z matky na dítě

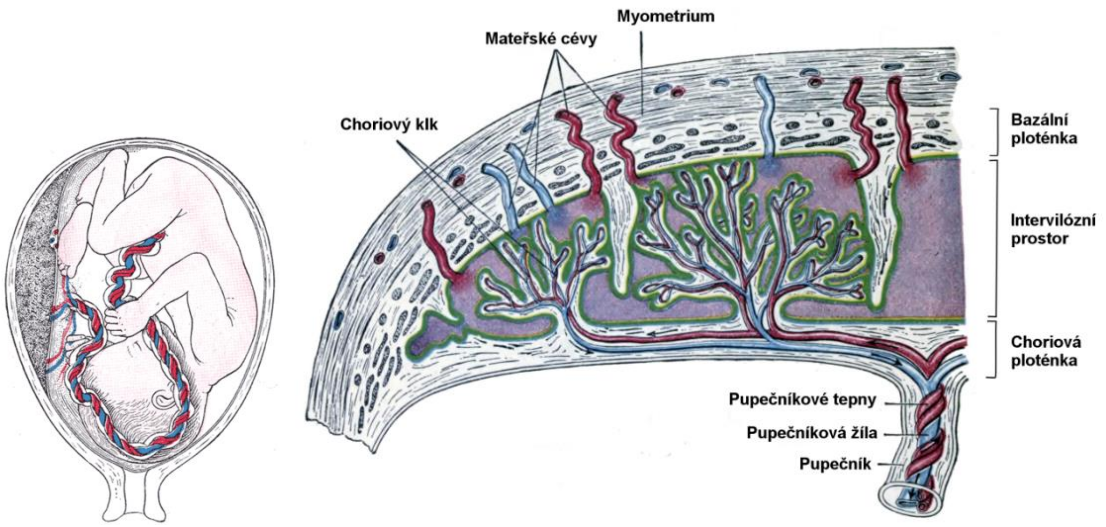
Řada studií potvrdila souvislost mezi snížením množství HIV RNA v krvi matky v důsledku užívání antiretrovirotik a pravděpodobností přenosu viru na její dítě [4]. Nicméně i u žen, u nichž bylo množství HIV RNA v krvi tak nízké, že jej podání léčiv už nedokázalo ovlivnit, byl prokázán benefit léčby a riziko MTCT viru bylo ještě dále sníženo [12]. Za tento přídatný protektivní mechanismus se považuje účinek antiretrovirotik v samotném fetálním oběhu, kdy léčivo po přechodu přes placentu chrání plod před nákazou virem a to nejen během kritické poslední fáze těhotenství a porodu [6]. Z výsledků klinických studií jednoznačně vyplývá, že kombinovaná antiretrovirální terapie (cART, z angl. combination antiretroviral therapy), tedy kombinace tří a více antiretrovirotik, podávána těhotným ženám co nejdelší dobu (ideálně ještě před otěhotněním) spolu s podáním antiretrovirotik novorozenci a následným vyvarováním se kojení představuje nejúčinnější léčebnou strategii [6], která snižuje riziko přenosu pod jedno procento [13].

Z výše uvedeného vyplývá, že znalosti mechanismů přestupu antiretrovirotik přes placentu jsou zásadní pro nastavení optimální terapie HIV pozitivních těhotných žen a pro pochopení profylaktického působení jednotlivých antiretrovirotik v MTCT.

3.3 Placenta

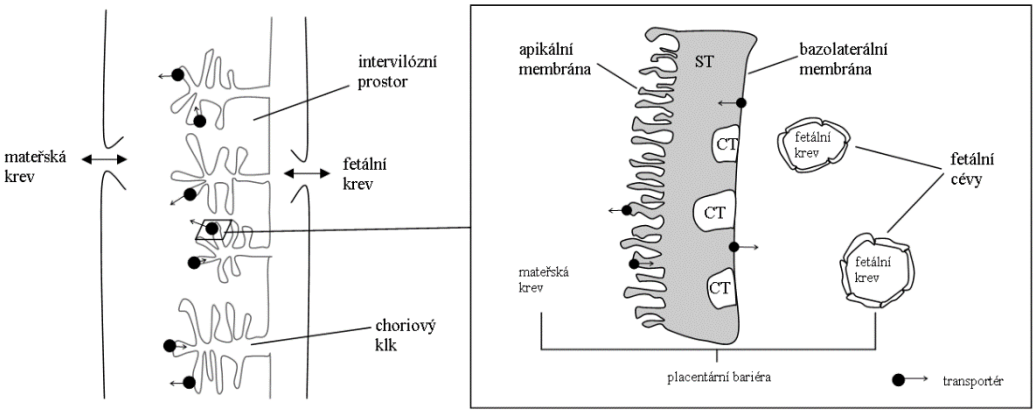
Placenta je orgán diskovitého tvaru, v němž se nachází v těsné blízkosti krevní oběhy plodu a matky a který zajišťuje funkční propojení mezi těmito dvěma organismy. Kromě zprostředkování látkové výměny a výživy plodu má placenta funkci endokrinní a imunitní a v neposlední řadě i protektivní, kdy limituje distribuci cizorodých látek z matky do plodu [14].

Vývoj placenty začíná vnořením blastocysty do děložní stěny, kdy dochází k fúzi fetální a mateřské tkáně. Během celého těhotenství pak prochází tento orgán významnými strukturálními i funkčními změnami. Fetální část zralé placenty představuje choriová ploténka (*chorion frondosum*, která drží pupečníkové cévy a z nich vybíhající choriové klky), mateřskou část placenty potom tvoří bazální ploténka (*decidua basalis* vyrůstající z děložního endometria). Mezi choriovou a bazální ploténkou se nachází intervilózní prostor, ve kterém jsou choriové klky omývány cirkulující mateřskou krví [15]. Schematické znázornění struktury zralé placenty představuje obrázek 1.



Obrázek 1 Schematické znázornění struktury placenty. Placenta je tvořena bazální (mateřská) a choriovou (fetální) ploténkou, mezi kterými se nachází intervilózní prostor, v němž cirkuluje krev matky. Ta omývá choriové klky, ve kterých se nachází fetální cévy a kde tedy dochází k látkovému přenosu mezi matkou a plodem. Převzato a upraveno [16].

Přímému mísení fetální a mateřské krve zabraňuje **placentární bariéra**, která je tvořena vrstvou buněk trofoblastu pokrývající povrch choriových klků, pod ní umístěné tenké vrstvy vaziva a konečně endotelem fetálních cév [15]. Buňky trofoblastu postupně prochází diferenciací. Původně jasně ohraničené buňky cytotrofoblastu splývají a směrem do intervilózního prostoru vyrůstá vrstva neohraničeného mnohojaderného soubuní, tzv. syncytiotrofoblastu, který je v přímém kontaktu s mateřskou krví. Právě tato vrstva je považována za hlavní složku placentární bariéry, která svou strukturou a vlastnostmi řídí přenos látek mezi matkou a plodem [17]. Buňky syncytiotrofoblastu jsou jasně polarizované. Apikální membrána (orientovaná směrem do intervilózního prostoru) je tvořena lemem mikroklků, které významně zvětšují povrch trofoblastu. Bazolaterální strana (nasedlá na cytotrofoblast a orientovaná směrem k plodu) je hladká a klky postrádá [18]. Obě membrány se dále liší expresí enzymů, receptorů a transportérů, které se podílí na obousměrném přenosu endogenních i exogenních látek mezi matkou a plodem [14, 19, 20]. Schéma placentární bariéry je představeno na obrázku 2.



Obrázek 2 Schematické znázornění placentární bariéry. Polarizovaná vrstva syncytiotrofoblastu tvořící povrch choriových klků představuje funkční složku placentární bariéry mezi fetální a mateřskou krví. CT, cytotrofoblast; ST, syncytiotrofoblast. Převzato a upraveno [17].

3.4 Mechanismy přestupu léčiv přes placentu

Nejčastějším transportním mechanismem, jakým léčiva přechází do fetálního krevního oběhu je **pasivní difúze**. Během tohoto děje dochází k volnému přechodu léčiv přes placentu ve směru koncentračního gradientu. Míra přestupu bude záviset jak

na vlastnostech daného léčiva (fyzikálně-chemické vlastnosti léčiva, vazba léčiva na plazmatické bílkoviny) tak i na faktorech specifických pro tento typ bariéry (rozdílné pH ve fetální a mateřské krvi, rychlost průtoku krve placentou, velikost povrchu i šířka placentární bariéry, která se mění v průběhu gestace). Další možností přestupu léčiv přes placentu je **využití transportéru**, tedy transmembránového proteinu, který umožňuje přenos i takových látek, které by pasivní difúzí prošly jen velmi omezeně. Za minoritní formu transplacentárního transportu je považována **transcytóza**, která se uplatňuje např. během přenosu imunoglobulinů od matky do plodu, nicméně v rámci problematiky přestupu léčiv se jeví jako nedůležitá [21].

3.5 Metody studia transplacentárního přestupu léčiv

Součástí cART má být podle doporučení zveřejňovaného americkým NIH nejméně jedno antiretrovirotikum s vysokým transplacentárním přestupem [6]. Takové léčivo má mít F/M poměr (poměr mezi plazmatickými hladinami léčiva v pupečníkové a mateřské krvi) hodnotu 0,6 a vyšší. Tím je zajištěna terapeutická léková hladina v těle plodu, která je důležitá především během porodu, kdy hrozí největší riziko nakažení [6]. Tato metoda stanovení má však několik omezení. Předně, vypočítaný F/M poměr popisuje přestup léčiv během terminální fáze těhotenství a tato pozorování tak nemohou být jednoduše extrapolována na období časnějších fází gestace, kdy taktéž dochází k infekcím plodu. Žena ve většině případů dostává poslední dávku léčiva před porodem a hladiny léčiv jsou následně stanoveny v jednom časovém bodě, což dále limituje farmakokinetickou analýzu přestupu přes placentu. Při prezentaci F/M poměru se také často nebere v potaz, zda bylo léčivo podáno samostatně či případně jaké kombinace antiretrovirotik bylo součástí. Proto vliv zároveň užívaných antiretrovirotik (ale i jiných současně podávaných léčiv) na tento poměr nemusí být odhalen.

Pro doplnění informací o transplacentárním přestupu antiretrovirotik je proto nezbytné využívat i dalších experimentálních metod, které mohou přispět k objasnění dané problematiky. Pro studium transportních mechanismů podílejících se na distribuci konkrétních léčiv mezi matkou a plodem byla vyvinuta řada metod, které využívají buňky či celé tkáně lidských placent (perfúzní experimenty, experimenty prováděné na buňkách izolovaného trofoblastu, placentárních fragmentech a vezikulech, buňkách odvozených od choriokarcinomu placenty), dále zvířecí modely, včetně experimentů na transgenních zvířatech, tkáních a buňkách a také počítačové modelování. Všechny tyto

přístupy mají své výhody a omezení a pouze jejich vzájemnou kombinací a detailním studiem placentárního přestupu antiretrovirotik na více úrovních je možné určit všechny faktory, které jej ovlivňují [22]. Charakteristiky vybraných metod, které byly použity v této dizertační práci, jsou podány v tabulce 1, další metodiky viz přehledový článek [22].

Tabulka 1 Vybrané metody studia role lékových efluxních transportérů v transplacentární farmakokinetice [22, 23].

Model	Výhody	Nevýhody
Buněčné linie transdukované lidskými transportéry (MDCKII, MEF3.8)	Technicky nenáročný model Umožňuje stanovit, zda je testovaná látka substrát konkrétního transportéru	Odlišné expresní profily studovaných transportérů v porovnání s buňkami lidského trofoblastu
Duálně perfundovaná potkaní placenta	Umožňuje sledovat transport testované látky přes placentu v obou směrech na intaktním orgánu Shodná lokalizace, exprese a funkce hlavních potkaních placentárních ABC transportérů s lidskými umožňuje studovat jejich vliv na transplacentární farmakokinetiku Umožňuje studovat lékové interakce Umožňuje použití induktorů studovaných transportérů před začátkem experimentu	Technicky náročný model Mezidruhové rozdíly ve stavbě a funkci potkaní a lidské placentární bariéry Studium transportních mechanismů je omezené na zralou placentu
Fragmenty lidské placentární tkáně	Zachovaná struktura placentární tkáně Umožňuje studium transportu léčiv v různých fázích gestace	Neumožňuje stanovení transportu v jednom směru, ale pouze určuje uptake placentární tkání Heterogenita tkáně
Izolované vezikuly z lidského trofoblastu	Umožňuje charakteristiku studovaných transportérů přímo na bazální nebo apikální membráně trofoblastu Umožňuje studium transportu léčiv v různých fázích gestace	Neumožňuje stanovit podíl činnosti daného transportérů na celkovém přestupu testované látky přes placentu

3.6 Placentární transportéry léčiv

V rámci studia mechanismů přestupu léčiv přes placentu byla za určující faktor placentární bariéry donedávna pokládána především její mechanická složka, tedy prostá přítomnost buněčných vrstev oddělující oba krevní oběhy. Identifikace placentárních transportérů až v nedávných letech potvrdila význam těchto přenašečů v transplacentárním přenosu řady endogenních i exogenních látek a mezi nimi i mnohých léčiv [24, 25]. Placentární transportéry rozdělujeme do dvou velkých skupin, a to na ABC (z angl. ATP-Binding Cassette) a SLC (z angl. Solute Carriers) transportéry [17]. V této kapitole je podán stručný přehled transportérů, které se účastní přestupu léčiv přes placentu. Výběr je zúžen s ohledem na zaměření této dizertační práce. Přehled vybraných transportérů exprimovaných v syncytiotrofoblastu je dále shrnut na obrázku 3.

3.6.1 ABC transportéry

ABC transportéry tvoří skupinu proteinů schopných zprostředkovat aktivní transport svých substrátů přes buněčnou membránu i proti značnému koncentračnímu gradientu [26]. Tyto transportéry byly a doposud stále jsou intenzivně studovány pro svůj význam ve vývoji lékové rezistence v terapii nádorových onemocnění [27]. Kromě tohoto fenoménu byla jejich činnost popsána i ve zdravých tkáních a orgánech, jako jsou střevo, ledviny, játra či mozek, kde ABC transportéry ve většině případů jakožto **efluxní pumpy** modulují absorpci, distribuci a eliminaci svých substrátů [28, 29].

3.6.1.1 P-glykoprotein (ABCB1, MDR1)

P-glykoprotein (ABCB1) byl podobně jako ostatní ABC transportéry poprvé objeven v souvislosti se studiem mechanismu rezistence nádorových buněk vůči vysokým dávkám cytostatik. Právě první funkční studie prokazující závislost mezi expresí myšího P-glykoproteinu a mírou expozice plodů vůči teratogennímu antiparazitiku ivermektinu, které je substrátem tohoto transportéru, nasměrovala výzkum studia transplacentární farmakokinetiky směrem k ABC transportérům [30].

Exprese ABCB1 v lidské placentě byla potvrzena na genové i proteinové úrovni, a to během celého těhotenství [31]. ABCB1 je v syncytiotrofoblastu lokalizován na apikální straně membrány, kde je schopen pumpovat své substráty ven do intervilózního prostoru do krve matky [32, 33], což bylo prokázáno i na orgánové úrovni pomocí duálně perfundované lidské a potkaní placenty [34-38]. Substráty ABCB1 jsou obecně

strukturně a funkčně velmi různorodé látky; patří mezi ně zástupci řady skupin léčiv, jako jsou cytostatika, antibiotika, antiepileptika, antiemetika, antivirotika či antiarytmika, z nichž mnohá léčiva jsou používána i v těhotenství k léčbě matky, plodu či obou [17]. ABCB1 je dnes považován za nejvýznamnější ABC transportér, který ovlivňuje farmakokinetické děje a předpokládá se jeho významná úloha i v distribuci léčiv mezi matkou a plodem [39].

3.6.1.2 Breast Cancer Resistance Protein (BCRP, ABCG2)

Breast Cancer Resistance Protein (ABCG2) je spolu s ABCB1 v současnosti nejlépe prostudovaným transportérem exprimovaným v placentární bariéře (viz obsáhlé přehledové články, které shrnují poznatky k ABCB1 [31] a ABCG2 [23]). O ABCG2 je známo, že je exprimován na apikální straně syncytiotrofoblastu, kde se podílí na ochraně plodu efluxem svých substrátů z placenty zpět do krve matky [40, 41]. Kromě shodné lokalizace a funkce v placentě sdílí ABCG2 s ABCB1 poměrně širokou substrátovou specificitu, kdy mezi jeho substráty patří zástupci ze skupin léčiv cytostatik, antidiabetik, antibiotik nebo antivirotik [17].

Exprese ABCG2 je v organismu největší právě v placentární tkáni a zároveň bylo zjištěno, že je zde ABCG2 na úrovni mRNA exprimován ve vyšší míře než ABCB1 [41]. Knock-out tohoto myšího transportéru vedl ke zvýšené expozici plodů vůči jeho známým substrátům [42-45] a podobně jako u ABCB1 i u ABCG2 byla potvrzena funkčnost transportéru pomocí metody duálně perfundované lidské i potkaní placenty [46-48].

Mimo to ABCG2 ovlivňuje i vývoj samotného placentárního syncytia, chrání trofoblast před cytokiny vyvolanou apoptózou [49] a podílí se na přenosu endogenních steroidů přes placentu [50].

3.6.1.3 Multidrug Resistance-Associated Proteiny (ABCCs, MRPs)

Do rodiny Multidrug Resistance-Associated Proteinů (ABCCs) patří doposud devět členů (ABCC1-9) s překrývající se substrátovou specificitou a s různým poměrem zastoupení v jednotlivých tkáních [51]. Zprostředkovávají transport mnohých endogenních i exogenních látek a to především konjugátů s navázaným glutathionovým, glukuronidovým nebo sulfátovým zbytkem [26, 51]. Ovlivňují rovněž farmakokinetiku některých léčiv, mezi která řadíme zástupce ze skupin antivirotik, cytostatik,

hypolipidemik, antiarytmik či antiepileptik [17]. Dosavadní studie potvrdily expresi některých členů ABCCs na apikální i bazolaterální straně syncytiotrofoblastu. Zatímco ABCC2 se řadí po bok ABCB1 a ABCG2 mezi plod-ochraňující transportéry aktivní na apikální straně syncytiotrofoblastu, ABCC1, který je lokalizován bazolaterálně, bude naopak pohyb svých substrátů ve směru matka-plod usnadňovat [52-55].

Funkce ABCCs v placentě doposud nebyla plně objasněna. Nejlépe prozkoumaným transportérem je zde ABCC2, jehož inhibice vedla ke zvýšenému transplacentárnímu přestupu talinololu v materno-fetálním směru na modelu perfundované lidské placenty [56]. Exprese dalších ABCCs byla detekována v lidské i potkaní placentě [57, 58]. Patří mezi ně i ABCC5 a to na převážně na bazolaterální membráně syncytiotrofoblastu [59], nicméně stejně jako v případě dalších členů této podskupiny ABC transportérů, je i u něj potřeba více dat ke zjištění jejich úlohy v transplacentární kinetice léčiv.

3.6.2 SLC transportéry

Tato velká transportérová nadrodina má více než 300 zástupců, jejichž exprese byla popsána v různých orgánech lidského těla; především se jedná o ledviny, játra, střevo, mozek, placentu, plíce či varlata [57]. SLC transportéry jsou schopné přenášet celou řadu strukturně rozličných látek a fungovat jako transportéry **influxní** (do buňky), **efluxní** (z buňky) či **ekvilibrativní** (obousměrné). V placentě umožňují především pasivní (na energii nezávislý) uptake látek, jejichž fyzikálně-chemické vlastnosti jim neumožňují volný přestup přes buněčnou membránu [17]. SLC transportéry jsou významné v řadě fyziologických procesů, protože svou činností umožňují translokaci důležitých endogenních látek jako jsou nukleosidy, aminokyseliny, cukry či hormony a zároveň u nich byly popsány i interakce s léčivy, které vykazují strukturní podobnost s přirozenými substráty těchto transportérů [60].

V rámci studia transplacentárního transportu antiretrovirotik je pozornost věnována především studiu interakcí těchto léčiv s transportéry pro přenos organických kationtů (**OCTs**, z angl. organic cation transporters) a organických aniontů (**OATs**, z angl. organic anion transporters a **OATPs**, z angl organic anion-transporting polypeptides), **MATE** (z angl. Multidrug and Toxin Extrusion Proteins), ekvilibrativními nukleosidovými transportéry (**ENTs**, z angl. equilibrative nucleoside

transporters), a dalšími [61]. Stručné charakteristiky nejdůležitějších zástupců z rodiny SLC transportérů v placentě jsou podány v následující podkapitole.

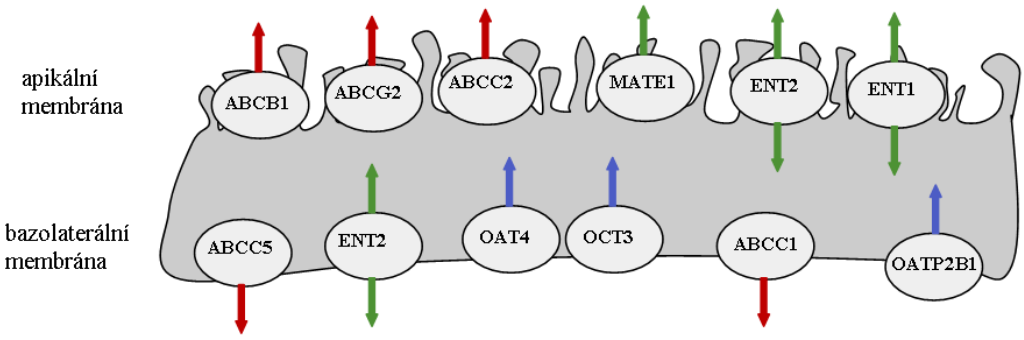
3.6.2.1 SLC transportéry podílející se na transportu antiretrovirotik

OCTs jsou polyspecifické transportéry, jejichž substráty jsou především organické kationty o nízké molekulové hmotnosti a které svou činností ovlivňují řadu fyziologických funkcí i patofyziologických procesů [57]. Nejlépe popsaným zástupcem v placentě je OCT3 exprimovaný na bazolaterální membráně trofoblastu, kde je elektrochemickým gradientem poháněn k oboustranné výměně kladně nabitých molekul [62]. Kromě endogenních látek jako jsou katecholaminy či acetylcholin jsou OCTs schopny rozpoznat zástupce z různých lékových skupin včetně antidepresiv či antiarytmik [17, 57].

Transportéry **MATE** přenáší přes buněčnou membránu řadu strukturně odlišných látek s většinou kladným nábojem ve své molekule [63]; v placentě byla dosud popsána exprese zástupců MATE1 a MATE2 [64]. Mezi klinicky významné substráty se řadí některá antiarytmika, antidepresiva nebo cytostatika [17, 57]. Právě součinnost MATE1 na apikální straně trofoblastu s OCT3 exprimovaným na protější straně placentární bariéry umožňuje vychytávání takových látek z fetální cirkulace zpět do krve matky. Tento typ vektoriálního transportu byl potvrzen zatím pouze na modelu potkaní placenty [65, 66], ale pravděpodobně probíhá i na placentě lidské, podobně jako v dalších orgánech, kde je spolupráce MATE/OCT velmi dobře prostudovaná [67-69].

OATs umožňují transport organických aniontů a podobně jako OCTs rozeznávají široké spektrum látek [57]. Z léčiv jsou to kromě antiretrovirotik také některá antihypertenziva, antidiabetika, či diuretika [70]. V placentě byla prokázána exprese OAT4 a to na bazolaterální membráně [71], kde jsou spolu s OATP2B1 (zástupcem ze skupiny **OATPs)** dalšími transportéry, u kterých se předpokládá schopnost vychytávat antiretrovirotika z fetální cirkulace [61].

Poslední skupinou SLC transportérů v placentě, u které se předpokládá ovlivnění distribuce antiretrovirotik, tvoří **ENTs**. Konkrétně se jedná o ENT1 a ENT2, jejichž úloha spočívá v translokaci endogenních nukleosidů [72, 73] a proto se předpokládá jejich účast na transportu těch antiretrovirotik, která jsou od nukleosidů strukturně odvozená. Tato problematika je v současnosti naším týmem rovněž intenzivně studovaná.



Obrázek 3 Schematické znázornění vybraných transportérů v syncytiotrofoblastu. ABCB1, P-glykoprotein; ABCG2, Breast Cancer Resistance Protein; ABCC2, Multidrug Resistance-Associated Protein 2; ABCC5, Multidrug Resistance-Associated Protein 5; MATE1, Multidrug and Toxin Extrusion Protein 1; ENT1, ekvilibrativní nukleosidový transportér 1; ENT2, ekvilibrativní nukleosidový transportér 2; OCT3, transportér pro přenos organických kationtů 3; OAT4, transportér pro přenos organických aniontů 4; OATP2B1, organické anionty transportující polypeptid 2B1. Převzato a upraveno dle [17] a [74].

3.7 Antiretrovirotika používaná k léčbě HIV pozitivních těhotných žen a jejich interakce s placentárními ABC transportéry

Pokud HIV pozitivní žena ještě před otěhotněním nebyla léčena antiretrovirotiky, měla by s užíváním léčiv začít pro prevenci přenosu viru na dítě [5, 6]. V současnosti se doporučuje u těchto žen začít ihned, a to s farmakoterapií využívající cART, jaká je používána u dospělé populace. Ta zahrnuje kombinaci tří a více léčiv z různých farmakodynamických skupin tak, aby bylo zasaženo více fází životního cyklu HIV. Nejčastěji se jedná o kombinaci dvou léčiv ze skupiny nukleosidových/nukleotidových inhibitorů reverzní transkriptázy (NRTI, z angl. nucleoside/nucleotide reverse transcriptase inhibitor) spolu se dvěma zástupci inhibitorů proteázy (PI, z angl. protease inhibitor) nebo ne-nukleosidovým inhibitorem reverzní transkriptázy (NNRTI, z angl. non-nucleoside reverse transcriptase inhibitor). Léčiva z dalších farmakoterapeutických skupin jako jsou inhibitory fúze nebo inhibitory integrázy nejsou u těhotných žen v současnosti preferovány jako léky první volby; nicméně jejich spotřeba u těhotných žen stoupá a mohou být vhodnou alternativou u žen, u kterých selhala klasická cART léčba [6].

3.7.1 Antiretrovirotika jako substráty ABC transportérů

Řada klinicky významných antiretrovirotik vystupují jako substráty a/nebo inhibitory některého ABC transportéru [61]. Je proto odůvodněné předpokládat, že transport těchto léčiv přes placentu bude činností placentárních ABC transportérů také ovlivněn. Zajímavá situace pak nastává, pokud je podána kombinace více substrátů/inhibitorů (situace typická při užívání cART) kdy může dojít k vzájemným farmakokinetickým interakcím, které vedou k rozdílné distribuci léčiv mezi matkou a plodem. Lze tedy spekulovat, že expozice plodu vůči antiretrovirotikům se může lišit v závislosti na zvoleném typu cART.

Pokud budeme posuzovat jako hlavní ukazatel míry transplacentárního přestupu léčiv v době porodu jejich F/M poměr, pak zjistíme, že zástupci léčiv ze skupiny PIs přechází přes placentu v terminální fázi těhotenství jen velice omezeně [75, 76]. To může být do určité míry přičteno právě efluxním transportérům, které ovlivňují farmakokinetiku PIs [61]. Naproti tomu doposud získané F/M poměry klinicky významných NRTIs jsou obecně vyšší než je tomu u PIs [77-83]. Zároveň byl ale u NRTIs ale zjištěn poměrně vysoký rozptyl v naměřených hodnotách [80, 84, 85], což

naznačuje značné interindividuální rozdíly v distribuci léčiv a tím i možnou úlohu transportních mechanismů jiných než je pasivní difúze. Pro optimalizaci léčby a výběr cART je proto důležité shromáždit více dat z klinické i neklinické části výzkumu transplacentární farmakokinetiky.

3.7.2 Antiretrovirotika jako regulátory exprese ABC transportérů

Jak již bylo zmíněno, placenta je orgán, který se v průběhu gestace dynamicky vyvíjí. Celý proces těhotenství je řízen systémem regulací, které umožňují fyziologický vývoj plodu. Z výsledků nedávných studií vyplývá, že tyto regulace se podílí i na řízení exprese a funkce placentárních transportérů, kdy u obou nejvýznamnějších transportérů, ABCB1 i ABCG2, byly zjištěny změny exprese v závislosti na fázi gestace [23, 31]. Kromě těchto fyziologických změn bylo doposud identifikováno i několik patologických faktorů, které mohou ovlivnit funkce placentárních ABC transportérů. Předpokládá se vliv zánětlivých procesů doprovázejících onemocnění matky jako je gestační diabetes, pre-eklampsie či infekční onemocnění [22] pravděpodobně i včetně HIV infekce [86]. Xenobiotika obecně vykazují schopnost ovlivnění exprese transportérů a to zejména při opakovaném užívání. *In vitro* data jako nejpřístupnější metoda k studiu této problematiky již prokázala indukční vliv antiretrovirotik ze skupin PIs, NRTIs i NNRTIs na expresi ABC transportérů [61, 87, 88]. Potenciál antiretrovirotik ovlivnit expresi daných transportérů je o to zajímavější, že léčba cART je indikována po celou dobu těhotenství a zvýšená aktivita ABC transportérů tak časem opět může změnit distribuci léčiv a to nejen mezi matkou a plodem. Tato data jsou tak klinicky velmi cenná a mohou přispět k optimalizaci terapie HIV infikovaných žen.

3.7.3 Antiretrovirotika studovaná v rámci této dizertační práce

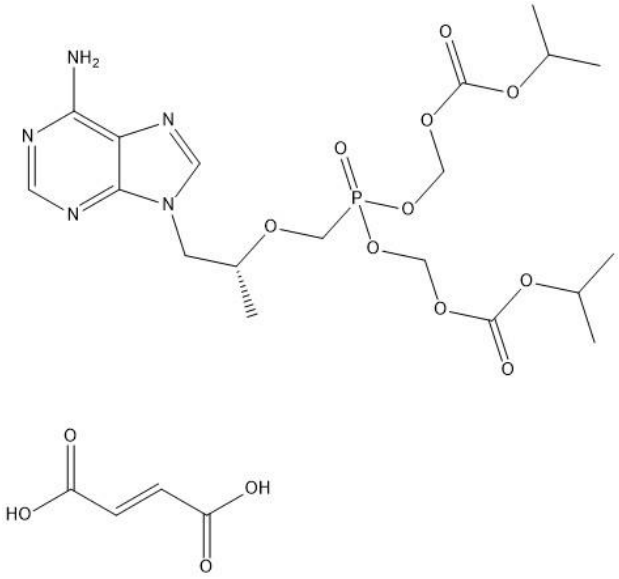
Všechna antiretrovirotika, která byla studována v rámci této dizertační práce, jsou používána u těhotných žen. Tato léčiva byla vybrána na základě jejich klinického významu a potenciálu interakcí s placentárními efluxními transportéry.

3.7.3.1 Tenofovir

Tenofovir (TFV) je purinové NRTI, které je kromě terapie infekce HIV používáno i u těhotných žen s infekcí hepadnaviry, zjm. u hepatitidy B [89]. Od svého uvedení na trh (2001) se TFV postupně stalo jedním z nejpoužívanějších léčiv v rámci prevence a terapie HIV infekce a jeho spotřeba roste i u těhotných žen [90, 91], kde je doporučováno jako léčivo první volby v rámci léčby samotné infekce i prevence MTCT

HIV [5, 6]. V současnosti je k dispozici ve formě proléčiva tenofoviru disoproxilu fumarátu (TDF), které zlepšuje biodostupnost TFV po perorálním podání a také jeho buněčný uptake [92, 93]. Existuje řada přípravků s fixní kombinací TDF s dalšími antiretrovirotiky; největší klinická zkušenost je s režimem TDF/ emtricitabin (*Truvada*) a TDF/emtricitabin/efavirenz (*Atripla*) [6]. TDF je poměrně dobře snášen těhotnými ženami [6, 94] nicméně jeho užívání je také spojeno s některými nežádoucími účinky na vyvíjející se plod, kdy se diskutuje hlavně o vlivu TDF na vývoj kostí [95] s dosud nejasným klinickým dopadem.

TFV je považováno za léčivo s vysokým placentárním přestupem, kdy F/M poměr dosahuje hodnot 0,6 - 1,0 [77, 78]. U obou molekul, TFV i TDF, vzniklo několik prací, které se věnovaly jejich interakcím s ABC transportéry. Na rozdíl od TFV, který neinteraguje s ABCB1 ani ABCC2 [96, 97], u TDF se předpokládá role ABCB1 substrátu [98, 99]. Komplexnímu zhodnocení vlivu ABCB1, ABCG2 a ABCC2 na transplacentární transport obou látek se věnuje článek I této dizertační práce. Vliv dlouhodobého podávání TFV na expresi vybraných ABC transportérů v potkaní placentě, ale i orgánech matky a plodu se dále věnuje článek IV.



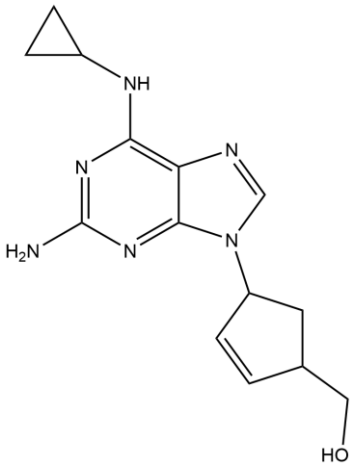
Obrázek 4 Tenofovir disoproxil fumarát

3.7.3.2 Abakavir

Abakavir je purinové NRTI, které je v klinické praxi od roku 1998. Z fixních kombinací je v současnosti k dispozici abakavir/lamivudin (*Epzicom*, *Kivexa*),

abakavir/lamivudin/AZT (*Trizivir)* a abakavir/lamivudin/dolutegravir (*Triumeq)* [6]. U 5 – 8 % žen se po podání abakaviru objevuje hypersenzitivní reakce; náchylní jsou pacienti s alelou HLA-B*5701 [100]. Proto se před začátkem léčby doporučuje provést genetický screening a pacientům s konkrétní alelou abakavir nepodávat [6]. Navzdory tomuto nežádoucímu účinku je abakavir v klinických studiích snášen těhotnými ženami velmi dobře [6, 101].

Jako lipofilní látka snadno proniká přes tělní bariéry včetně placentární [102], kdy F/M poměr je blízký jedné [80, 81]. Autoři několika studií s použitím různých *in vitro* modelů zjistili, že abakavir se chová jako substrát ABCB1 a ABCG2 [103-105], přičemž oba transportéry mají vliv i na distribuci abakaviru *in vivo* [103, 106]. Článek II této dizertační práce pak hodnotí roli transportérů ABCB1, ABCG2, ABCC2 a ABCC5 v transplacentární farmakokinetice tohoto léčiva.



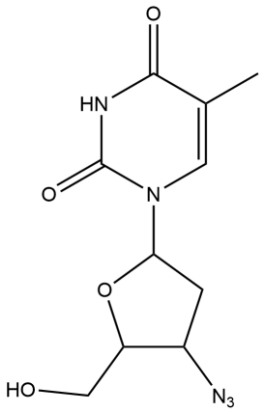
Obrázek 5 Abakavir

3.7.3.3 Zidovudin

AZT je pyrimidinové NRTI, které zůstává díky mnohaleté zkušenosti a příznivé ceně stále na předních místech v preskripci těhotným HIV pozitivním ženám [4]. AZT je k dispozici podobně jako další antiretrovirotika ve fixních kombinacích (spolu s lamivudinem v přípravku *Combivir* a dále ve složení AZT/lamivudin/abakavir jako *Trizivir)* i samostatně (*Retrovir*). V rámci levnějších preventivních režimů je pak AZT samostatně určený pro jednodávkovou aplikaci při porodu. Navzdory obecně dobré snášenlivosti během těhotenství je s jeho užíváním spojeno zvýšené riziko vzniku

hematologických a srdečních komplikací u dětí narozených matkám, které AZT užívaly v rámci prevence MTCT HIV [107-110].

AZT na konci těhotenství přestupuje přes placentu, jeho F/M poměr se pohybuje kolem jedné [80]. Co se týká doposud popsaných interakcí s hlavními ABC transportéry, pak AZT transport byl ovlivněn ABCG2 [111] a ABCCs [112]. Zatímco někteří autoři pozorovali eflux AZT zprostředkovaný transportérem ABCB1 [113], jiní jej vyloučili [103]. Objasněním role hlavních efluxních transportérů v transplacentárním přestupu AZT měl za cíl článek III této dizertační práce.



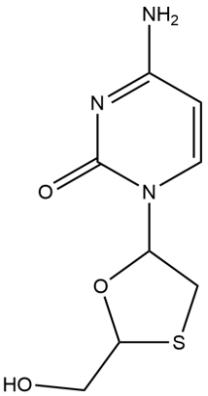
Obrázek 6 Zidovudin

3.7.3.4 Lamivudin

Lamivudin je pyrimidinové NRTI, které je v současnosti jedno z nejužívanějších léčiv v rámci terapie infekce HIV [4]. Vděčí za to oblibě fixní kombinace s AZT (*Combivir*) jako nejdéle užívaným léčivem v rámci prevence MTCT HIV. Díky svému příznivému bezpečnostnímu profilu a dobré snášenlivosti je u těhotných žen doporučován jako jeden z léků první volby [5]. Dále má své místo i v terapii virové hepatitidy B [6].

Lamivudin ve vysoké míře přestupuje přes placentu; jeho F/M poměr se pohybuje kolem jedné [80, 84]. Role efluxních transportérů v transplacentární farmakokinetice lamivudinu zůstává neobjasněná. ABCB1 i ABCG2 sice ovlivnily transport lamivudinu přes buněčnou monovrstvu *in vitro*, což naznačuje, že je látka substrátem obou transportérů, nicméně autoři obou prací zpochybňují význam ABCB1 i ABCG2 ve farmakokinetice tohoto léčiva [113, 114]. Interakcím lamivudinu

s efluxními transportéry a jejich vlivu na transplacentární transport lamivudinu samostatně i v kombinaci s AZT se věnuje článek III a částečně článek V této dizertační práce.

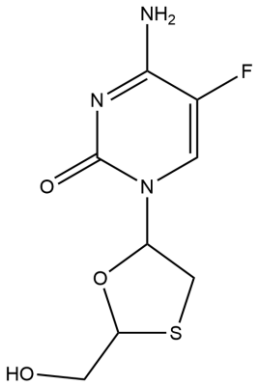


Obrázek 7 Lamivudin

3.7.3.5 Emtricitabin

Emtricitabin je novější pyrimidinové NRTI (schváleno FDA v roce 2003), které je podobně jako tenofovir a lamivudin účinné proti infekcím virem HIV a virem hepatitidy B [115]. Emtricitabin je spolu s TFV určen pro prevenci přenosu viru HIV mezi dospělými osobami a jeho význam stoupá i v rámci terapie těhotných žen [116, 117]. Fixní kombinace emtricitabinu obsahují vždy také TDF – tato dvojkombinace je k dostání v přípravku *Truvada.* K TDF/emtricitabinu se dále přidávají léčiva efavirenz (*Atripla*) nebo nověji v letech 2012 a 2013 schválené kombinace s elvitegravirem a kolbicistatem (*Stribild*) a rilpivirinem (*Complera*) [6].

Emtricitabin přestupuje přes placentu s F/M poměrem pohybujícím se okolo jedné [79, 82, 83]. O interakcích emtricitabinu s ABC efluxními transportéry není v současnosti mnoho informací, výjimku tvoří popsané interakce s ABCCs [118-120] a ABCB1 [121]. V článku IV této dizertační práce jsme se zabývali tím, zda dlouhodobá aplikace emtricitabinu v průběhu gestace může mít vliv na expresi vybraných efluxních ABC transportérů na potkaním modelu.

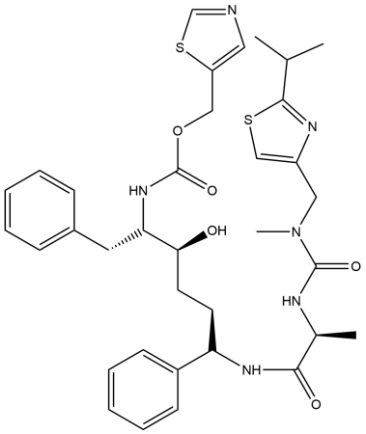


Obrázek 8 Emtricitabin

3.7.3.6 Ritonavir

Ritonavir je jedním z nejstarších zástupců ze skupiny léčiv PIs. Jeho uvedení na trh v 90. letech minulého století v době, kdy bylo k dispozici k léčbě HIV infekce pouze několik NRTIs, umožnilo zavést do praxe první vysoce účinné kombinace antiretrovirotik. Stejně jako ostatní zástupci této skupiny, patří i ritonavir mezi léčiva s potenciálem lékových interakcí jako prokázané substráty/inhibitory efluxních transportérů a enzymu CYP 450 [61]. V současnosti se ritonavir v rámci cART nepoužívá pro svůj vlastní farmakologický účinek, ale přidává se jako booster – inhibitor efluxních transportérů a enzymu CYP 450 pro pozitivní ovlivnění farmakokinetiky dalších PIs. Pro použití v těhotenství je k dispozici ve fixní kombinaci s lopinavirem (*Kaletra*); jako samotný (*Norvir*) lze potom přidat do kombinace se všemi ostatními PIs. Z nežádoucích účinků u těhotných žen je pak třeba vzít do úvahy především zvýšené riziko vzniku poruch glukózové tolerance a gestačního diabetu [6].

Transplacentární farmakokinetiku ritonaviru jsme studovali se zaměřením na roli ritonaviru jako substrátu a inhibitoru efluxních transportérů ABCB1, ABCG2 a MATE1. Tato publikace je v současnosti připravována a nebyla zahrnuta do této dizertace.



Obrázek 9 Ritonavir

4 Cíle práce

Náplní této dizertační práce bylo studium interakcí nejvýznamnějších antiretrovirotik používaných v péči o těhotné HIV pozitivní ženy s hlavními placentárními transportéry, u nichž je známo, že ovlivňují distribuci léčiv mezi matkou a plodem.

Konkrétně se jednalo o splnění těchto cílů:

i. popsat interakce TFV i jeho proléčiva TDF s transportéry ABCB1, ABCG2 a ABCC2 za použití *in vitro* transportních experimentů s MDCKII buňkami transdukovanými lidskými transportéry a *in situ* metody duálně perfundované potkaní placenty

ii. popsat interakce abakaviru s transportéry ABCB1, ABCG2, ABCC2 a ABCC5 za použití *in vitro* transportních experimentů s MDCKII buňkami transdukovanými lidskými transportéry, *in situ* metody duálně perfundované potkaní placenty a *ex vivo* akumulačních experimentů s fragmenty izolovanými z lidské placenty

iii. popsat interakce zidovudinu s transportéry ABCB1, ABCG2, ABCC2 a ABCC5 za použití *in vitro* transportních experimentů s MDCKII buňkami transdukovanými lidskými transportéry a *in situ* metody duálně perfundované potkaní placenty

iv. popsat vliv dlouhodobého užívání tenofoviru a emtricitabinu na expresi *Abcb1a, Abcb1b* a *Abcg2* pomocí kvantitativní RT-PCR analýzy s využitím *in vivo* modelu březího potkana

v. popsat interakce lamivudinu s transportéry ABCB1, ABCG2, ABCC2 a MATE1 za použití *in vitro* transportních experimentů s MDCKII buňkami transdukovanými lidskými transportéry, akumulačních experimentů na buňkách MEF3.8, *in situ* metody duálně perfundované potkaní placenty a *ex vivo* akumulačních studií na vezikulech z izolované mikrovilózní membrány lidského trofoblastu (MVM vezikuly, z angl. microvillous plasma membrane vesicles)

5 Seznam prací a podíl kandidátky na jednotlivých publikacích

Tato dizertační práce je předkládána jako komentovaný soubor prací. Čtyři práce byly otištěny v zahraničních časopisech s IF a jedna práce je v současné době v recenzním řízení. Všechny manuskripty jsou původní experimentální práce zaměřené na popis a vyhodnocení interakcí klinicky relevantních antiretrovirotik s placentárními transportéry.

Kandidátka je první autorkou tří prací (I, II a III) a spoluautorkou dvou prací (IV a V). Její podíl na jednotlivých publikacích je následující:

I. Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta

- praktické provedení všech perfúzních experimentů, analýza a prezentace dat a podíl na sepsání manuskriptu

II. Effect of drug efflux transporters on placental transport of antiretroviral agent abacavir

- praktické provedení všech perfúzních a části buněčných experimentů, podíl na experimentech využívajících akumulace na placentárních fragmentech, analýza dat a sepsání manuskriptu

III. Role of ABCB1, ABCG2, ABCC2 and ABCC5 transporters in placental passage of zidovudine

- praktické provedení všech perfúzních a části buněčných experimentů, analýza dat a sepsání manuskriptu

IV. Long-term administration of tenofovir or emtricitabine to pregnant rats; effect on *Abcb1a*, *Abcb1b,* and *Abcg2* expression in the placenta and in maternal and fetal organs

- praktické provedení studií srovnávající biodostupnost testovaných látek, podíl na aplikaci testovaných látek zvířatům, odběru orgánů a expresní analýze, revize manuskriptu

V. Interaction of lamivudine with ABC and SLC transporters *in vitro* and *in vivo*: role in placental transport

- praktické provedení části perfúzních experimentů, podíl na provedení experimentů využívajících MVM vezikuly z lidského trofoblastu, podíl na analýze dat a revize manuskriptu

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7 Jednotlivé práce a jejich komentáře

7.1 Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta

Zuzana Neumanová, Lukáš Červený, Martina Čečková, František Štaud

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TFV je v současnosti jedno z nejpoužívanějších léčiv v rámci prevence a terapie HIV infekce u těhotných žen. Cílem této práce bylo zjistit, zda TFV a/nebo jeho proléčivo TDF je substrátem ABCB1, ABCG2 a ABCC a zhodnotit, zda mají tyto transportéry úlohu v transplacentárním přestupu obou molekul.

Za pomocí *in vitro* transportních experimentů s MDCKII buňkami transdukovanými lidskými transportéry jsme zjistili, že TDF je substrátem transportérů ABCB1 a ABCG2, ale neinteraguje s ABCC2. Rozdíly v transplacentární clearance TDF, které jsme získali využitím *in situ* metody duálně perfundovaní potkaní placenty indikovaly aktivní transport TDF ve feto-maternálním směru. Pomocí uzavřeného perfuzního systému a za použití specifického inhibitoru jsme pak potvrdili role ABCB1 a ABCG2 v transplacentárním transportu TDF.

Na druhou stranu TFV nevykazoval chování substrátu žádného z testovaných transportérů *in vitro* ani *in situ*. Nízké hodnoty transplacentární clearance ukazovaly na omezený přestup TFV přes placentu v obou směrech.

Z výše uvedených výsledků vyvozujeme, že transport obou látek, TFV a TDF, z matky do plodu je omezen. V případě TFV je transport přes placentu limitován v důsledku fyzikálně-chemických vlastností této molekuly, zatímco u TDF je snížen činností ABC transportérů ABCB1 a ABCG2.

Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta

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Objective and design: Tenofovir (TFV) is used in pregnant women as a part of combination antiretroviral treatment to prevent mother-to-child transmission of HIV infection. We aimed to detect whether TFV and/or its prodrug, tenofovir disoproxil fumarate (TDF), are substrates of ATP-binding cassette (ABC) transporters that are functionally expressed in the placenta, namely P-glycoprotein (ABCB1/MDR1), Breast Cancer Resistance Protein (ABCG2/BCRP) and Multidrug Resistance-Associated Protein 2 (ABCC2/MRP2). We employed in-vitro cell-based assays and in-situ animal model to assess possible role of the efflux transporters in transplacental pharmacokinetics of TFV and TDF.

Methods: In-vitro transport assays were performed in MDCKII cells transduced with human ABCB1, ABCG2 or ABCC2. To quantify the effect of these transporters on TFV/TDF transplacental passage, we employed the in-situ model of dually perfused rat term placenta in open and closed setup.

Results: In-vitro assays revealed that TDF is a dual substrate of ABCB1 and ABCG2 but not of ABCC2. In contrast, TFV transport was not influenced by any of these transporters. Applying concentration-dependent studies and selective inhibitors, we further confirmed these findings *in situ* on the organ level; both ABCB1 and ABCG2 limited mother-to-fetus transfer of TDF whereas TFV transplacental passage was not affected by these ABC transporters.

Conclusion: We propose limited mother-to-fetus transport of both TFV and TDF. While placental transport of TFV is restricted passively, by physical-chemical properties of the molecule, mother-to-fetus passage of TDF is actively hindered by placental ABCB1 and ABCG2 transporters, pumping this compound from trophoblast back to maternal circulation.
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Introduction

An estimated 1.5 million pregnant women were living with HIV in 2011 [1]. According to the WHO, all infected women, regardless of their clinical stage, should receive combination antiretroviral treatment throughout gestation to prevent HIV mother-to-child transmission (MTCT) [2]. Together with other evidence-based interventions, antiretroviral pharmacotherapy in pregnancy reduces the percentage of HIV-positive infants from 20–45% to 1–2% [3].

Tenofovir (TFV), a nucleotide reverse transcriptase inhibitor, represents a backbone of combination anti-HIV

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therapy [4]. To improve its pharmacokinetic properties, TFV is administered in the form of disoproxil fumarate. Despite being classified as pregnancy category B drug, WHO has incorporated tenofovir disoproxil fumarate (TDF) into recent guidelines for prophylaxis of HIV MTCT [2] and its use in pregnancy tends to increase [5,6]. It must be stressed out that medication of pregnant women requires special attention to guarantee adequate and well tolerated therapy throughout gestation; among others, changes in pharmacokinetics during pregnancy [7] and quantification of drug transport across the placenta [8] need to be taken into account.

Several members of ATP-binding cassette (ABC) drug efflux transporters have been localized in the apical, maternal-facing membrane of the placenta where they pump their substrates from the trophoblast cells back to the maternal circulation, thus limiting permeation of substrate drugs from mother to fetus [8]. To date, the best-described ABC transporters in the placenta are: P-glycoprotein (ABCB1/MDR1) [9], Breast Cancer Resistance Protein (ABCG2/BCRP) [10] and Multidrug Resistance-Associated Protein 2 (ABCC2/MRP2) [11]. It has been well documented that these transporters affect transplacental passage of many clinically used compounds, including antiretrovirals [12]. Therefore, detailed knowledge on drug interactions with placental ABC transporters is required to complete their safety profile and to guarantee adequate and well tolerated medication of pregnant woman and her child [13]. Interactions of TFV and TDF with ABCB1 and ABCC2 have been investigated in several studies, however, providing inconsistent results depending on the method used [14–20]. To our knowledge, data clearly describing ABCG2-mediated transport of TFV or TDF is still lacking. In addition, influence of ABC transporters on transplacental passage of these compounds has not been evaluated so far.

In the present study, we employed the in-vitro model of MDCKII cells transduced with human ABC transporters to investigate whether TFV and/or TDF are substrates of human ABCB1, ABCG2 or ABCC2. Furthermore, using the model of in-situ perfused rat placenta we aimed to elucidate potential effect of these transporters on TFV/TDF passage from mother to fetus.

Methods

Reagents and chemicals

Tenofovir [TFV, (1R)-9-(2-Phosphonylmethoxypropyl)-adenine)] and tenofovir disoproxil fumarate [TDF; bis(isopropyloxycarbonyloxymethyl)9-(2-Phosphonyl methoxypropyl)-adenine] were kindly provided by Gilead Sciences, Inc. (Foster City, California, USA). Radiolabeled [adenine-2,8–3H]tenofovir ([^{3}H]TFV) and [adenine-8–3H] tenofovir disoproxil fumarate ([^{3}H]TDF) were purchased from Moravek Biochemicals (California, USA). Dual ABCG2 and ABCB1 inhibitor, GF120918, was kindly provided by GlaxoSmithKline (Greenford, UK). Indomethacin, nonselective inhibitor of ABCC(s), was purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Pentobarbital (Nembutal) was purchased from Abbott Laboratories (Abbott Park, Illinois, USA). All other chemicals were of analytical grade.

Cells

MDCKII (Madine-Darby Canine Kidney) parental cell line and MDCKII cells stably transduced with cDNA of human MDR1 (MDCKII-ABCB1), BCRP (MDCKII-ABCG2) or MRP2 (MDCKII-ABCC2) were obtained from Netherlands Cancer Institute (Dr A. Schinkel) and cultured in DMEM complete high-glucose medium with L-glutamine, supplemented with 10% fetal bovine serum.

Animals

Pregnant Wistar rats were purchased from Biotest Ltd. (Konarovice, Czech Republic) and were maintained in 12/12-h day/night standard conditions with water and pellets *ad libitum*. Experiments were performed on day 21 of gestation. Fasted rats were anesthetized with pentobarbital (Nembutal; Abbott Laboratories) in a dose of 40 mg/kg administered into the tail vein. All animal experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes.

Transport experiments *in vitro*

Transport assays were performed on microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, New York, New York, USA) as described previously [21]. MDCKII-parental and MDCKII cells expressing ABCB1, ABCG2 or ABCC2 were seeded at a density of 1.0×10^6 cells per well and cultured for 72 h to confluence, including daily medium replacement. One hour before starting the experiment, cells were washed with phosphate-buffered saline (37°C) and incubated with 2 ml of Opti-MEM (Invitrogen, Carslbad, California, USA) either alone or containing the dual ABCB1 and ABCG2 inhibitor GF120918 (2 μmol/l). The experiment was started by replacing the medium in the donor compartment (either apical or basolateral) with fresh Opti-MEM (37°C) containing the tested compound, TFV or TDF, or combination of TDF with GF120918 (2 μmol/l). Both TFV and TDF were traced by [^{3}H]TFV and [^{3}H]TDF, respectively, achieving the final activity of 0.04 μCi/ml. The lowest point of the concentration range (3.1 nmol/l for TFV and 33.3 nmol/l

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for TDF) was determined by the specific activity of radioisotopes required for analysis. Aliquots of 50 μl were collected at 2, 4 and 6 h from the acceptor compartment and radioactivity was measured by liquid scintillation counting (Tri-Carb 2900 TR Perkin Elmer). At the end of the experiment leakage of FITC-dextran was analysed and was accepted up to 1% per hour. The percentage of radioactivity appearing in the acceptor compartment relative to stock solution initially added to the donor compartment was calculated. Ratios of basal-to-apical to apical-to-basal translocation after 6-h incubation (r) were calculated as described elsewhere [21,22].

Dual perfusion of the rat placenta *in situ*

The method of dually perfused rat term placenta was used as described previously [23]. In brief, one uterine horn was excised and submerged in heated Ringer's saline. A catheter was inserted into the uterine artery proximal to the blood vessel supplying a selected placenta and connected with the peristaltic pump. Kreb's perfusion liquid containing 1% dextran was brought from the maternal reservoir at a rate 1 ml/min. The uterine vein, including anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighbouring ones by ligatures. The umbilical artery was catheterized by use of a 24-gauge catheter connected to the fetal reservoir and perfused at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner, and the selected fetus was removed. Before the start of each experiment, the fetal vein effluent was collected into preweighed glass vial to check for a possible leakage of perfusion solution from the placenta. In the case of leakage, the experiment was terminated. Maternal and fetal perfusion pressures were maintained at levels close to physiological values and monitored continuously throughout the perfusion experiments as described previously. At the end of experiment, placenta was perfused with radioactivity-free buffer for 10 min, excised from the uterine tissue, and dissolved in tissue solubilizer (Solvable; PerkinElmer Life and Analytical Sciences), and its radioactivity was measured to detect tissue-bound TFV or TDF.

Two types of perfusion systems were used in this study:

Open-circuit perfusion system was employed to study fetal-to-maternal (F > M) and maternal-to-fetal (M > F) clearances of TFV or TDF at various concentrations. TFV (50 nmol/l or 500 μmol/l) and TDF (50 nmol/l, 100 or 500 μmol/l) was added to either maternal (M>F studies) or fetal (F>M studies) reservoir immediately after successful surgery. After 5-min stabilization period the sample collection started (time 0). Fetal effluent was sampled into preweighed vials in 5 min interval, concentrations were measured radiometrically and transplacental clearance was calculated from all measured intervals as described below.

Closed-circuit (recirculation) perfusion system was employed to identify placental transporter(s) responsible for active transport of TDF from the fetal circulation. Both maternal and fetal sides of the placenta were infused with either nonsaturating (50 nmol/l) or saturating (500 μmol/l) concentrations of [^{3}H]TDF and after short-time stabilization period, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (250 μl) were collected every 10 min from the maternal and fetal reservoirs, and concentrations of [^{3}H]TDF were measured. This experimental setup ensures steady concentration on the maternal side of the placenta and enables investigation of fetal/maternal ratio; any net transfer of the substrate implies transport against a concentration gradient and provides evidence of active transport. To determine the effect of efflux transporters on placental passage of TDF, GF120918 (2 μmol/l), a dual inhibitor of ABCB1 and ABCG2 [24], or indomethacin (0.28 mmol/l), a nonspecific inhibitor of ABCCs [25], were added to both maternal and fetal reservoirs and the fetal/maternal concentration ratio at equilibrium was calculated.

Pharmacokinetic analysis of efflux transport activity in the placenta

Organ clearance concept was applied to quantify M > F and F > M transport of TFV and TDF in open-circuit perfusion system [23]. Averaged data from the intervals of 10–35 min were used for the following calculations. M > F transplacental clearance (Cl_{mf}) normalized to placenta weight was calculated according to Eq. (1).

$$Cl_{mf} = \frac{C_{fv} \times Q_f}{C_{ma} \times W_p}$$

where C_{fv} is drug concentration in the umbilical vein effluent, Q_f is the umbilical flow rate, C_{ma} is concentration in the maternal reservoir, and W_p is the wet weight of the placenta. F > M transplacental clearance (Cl_{fm}) was calculated according to Eq. (2).

$$Cl_{fm} = \frac{(C_{fa} - C_{fv})Q_f}{C_{fa} \times W_p}$$

where C_{fa} is drug concentration in the fetal reservoir entering the perfused placenta via the umbilical artery.

Statistical analysis

In in-vitro cell-based studies, two-side unpaired Student's t-test was employed to compare apical-to-basal translocation and basal-to-apical-translocation at time point 6 h. In in-situ placenta perfusion studies, statistical significance was examined by two-side unpaired Student's t-test or one-way ANOVA followed by Bonferroni's test. All data were assessed using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, California, USA).

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Results

TFV transport across MDCKII parental and ABCB1, ABCG2, ABCC2-overexpressing cells

We first determined transepithelial transport of [^{3}H]TFV at the concentration of 3.1 nmol/l through the monolayers of parental and ABCB1, ABCG2 or ABCC2-overexpressing cells. No asymmetry in basal-to-apical vs. apical-to-basal transport of TFV was observed in MDCKII parental cells ($r = 0.90$). Transports across MDCKII-ABCB1, ABCG2 and ABCC2 monolayers were equivalent to that in parental cell line ($r = 0.89$, 0.79 and 0.89, respectively) (Table 1). These findings show that TFV is not a substrate of any of the transporters investigated.

TDF transport across MDCKII parental and ABCB1, ABCG2, ABCC2-overexpressing cells

Transepithelial transport of [^{3}H]TDF through MDCKII parental and ABCB1, ABCG2, ABCC2-overexpressing cells was measured at a concentration of 33.3 nmol/l. In the parental cells, basal-to-apical/apical-to-basal transport ratio (r) of 2.38 was observed likely resulting from activity of endogenous canine transporters as reported previously [22]. Compared with MDCKII parental cells, significantly larger basal-to-apical/apical-to-basal ratios were observed in ABCB1 and ABCG2-overexpressing cells ($r = 5.47$ and 6.24, respectively) but not in ABCC2 cells ($r = 3.06$). Increase in TDF concentration (10 μmol/l) significantly reduced this ratio in both ABCB1 and ABCG2 cells ($r = 4.56$ and $r = 4.78$, respectively) indicating partial saturation of both transporters. Furthermore, addition of a dual ABCB1 and ABCG2 inhibitor, GF120918 (2 μmol/l), completely abolished the asymmetry in translocation of TDF in respective cell lines at both concentrations tested (33.3 and 10 μmol/l) reaching transport ratio values of approximately 1. These findings demonstrate that TDF is a substrate of human ABCB1 and ABCG2 but not of ABCC2 (Table 2).

Open circuit perfusion experiments: effect of inflow concentrations on transplacental clearance of TFV and TDF

The maternal or fetal side of the placenta was infused with various concentration of TFV (50 nmol/l or 500 μmol/l) or TDF (50 nmol/l, 100 or 500 μmol/l). No statistically significant differences between M>F and F>M clearances were observed at either low or high concentration of TFV, suggesting linear mechanism in transplacental transport of this compound. On the contrary, increase in TDF concentration resulted in significant changes in transplacental clearances in both M>F and F>M directions, confirming involvement of a capacity-limited transport mechanisms (Fig. 1). Less than 5% of TFV or TDF dose was detected in the placenta after perfusion experiments, suggesting limited tissue binding and negligible effect on clearance calculation.

Closed circuit perfusion experiments: effect of concentration and inhibitors on TDF transport across the placenta

To identify placental transporter(s) responsible for elimination of TDF from the fetal circulation, both sides of placenta were perfused with low nonsaturating concentration of TDF in closed circuit experimental setup in either absence or presence of inhibitors. In the absence of inhibitors, we observed significant decrease in TDF concentration in fetal perfusate, confirming active transport of this compound from fetal to maternal side of the placenta against concentration gradient. This decline was fully blocked by coinfusion with 500 μmol/l TDF confirming saturable transport. Furthermore, transport of TDF from fetus to mother was significantly blocked by GF120908 (2 μmol/l), whereas co-administration of indomethacin (0.28 mmol/l) did not show any effect (Fig. 2).

Discussion

Although TFV is categorized as a pregnancy B drug by FDA, it is frequently used in the treatment of pregnant women with HIV infection. Nevertheless, transport of this compound, and its prodrug TDF, across the placenta from mother to fetus and the role of placental ABC drug efflux transporters in this event have not been systematically investigated to date. In this study we employ both in-vitro and in-situ experimental approaches to characterize interactions of TFV and TDF with the best-described ABC transporters localized in the placenta, that is ABCB1, ABCG2 and ABCC2, and to

Table 1. Tenofovir transport in MDCKII parental and ABCB1, ABCG2 and ABCC2-overexpressing cells.

Cell line (MDCKII)	TFV concentration	Transport ratio (basal-to-apical/apical-to-basal)
Parental	3.1 nmol/l	0.90 ± 0.05
ABCB1	3.1 nmol/l	0.89 ± 0.03
ABCG2	3.1 nmol/l	0.79 ± 0.08
ABCC2	3.1 nmol/l	0.89 ± 0.05

Data points are means ($n = 3$) ± SD, Student's t-test was employed to compare apical-to-basal translocation and basal-to-apical translocation. TFV, tenofovir; ABCB1, P-glycoprotein; ABCG2, Breast Cancer Resistance Protein; ABCC2, Multidrug Resistance-Associated Protein 2.

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Table 2. Tenofovir disoproxil fumarate transport in MDCKII parental and ABCB1, ABCG2 and ABCC2-overexpressing cells.

Cell line (MDCKII)	TDF concentration (with or without inhibitor)	Transport ratio (basal-to-apical/apical-to-basal)
Parental	33.3 nmol/l	$2.38 \pm 0.04^*$
ABCB1	33.3 nmol/l	$5.47 \pm 0.76^*$
	33.3 nmol/l + GF120918 (2 μmol/l)	0.75 ± 0.02
	10 μmol/l	$4.56 \pm 0.12^*$
	10 μmol/l + GF120918 (2 μmol/l)	0.96 ± 0.03
ABCG2	33.3 nmol/l	$6.24 \pm 0.57^*$
	33.3 nmol/l + GF120918 (2 μmol/l)	1.25 ± 0.12
	10 μmol/l	$4.78 \pm 0.07^*$
	10 μmol/l + GF120918 (2 μmol/l)	0.67 ± 0.06
ABCC2	33.3 nmol/l	$3.06 \pm 0.99^*$

Data points are means ($n = 3$) ± SD, Student's *t*-test was employed to compare apical-to-basal translocation and basal-to-apical translocation. $^*P < 0.001$. TDF, tenofovir disoproxil fumarate; ABCB1, P-glycoprotein; ABCG2, Breast Cancer Resistance Protein; ABCC2, Multidrug Resistance-Associated Protein 2

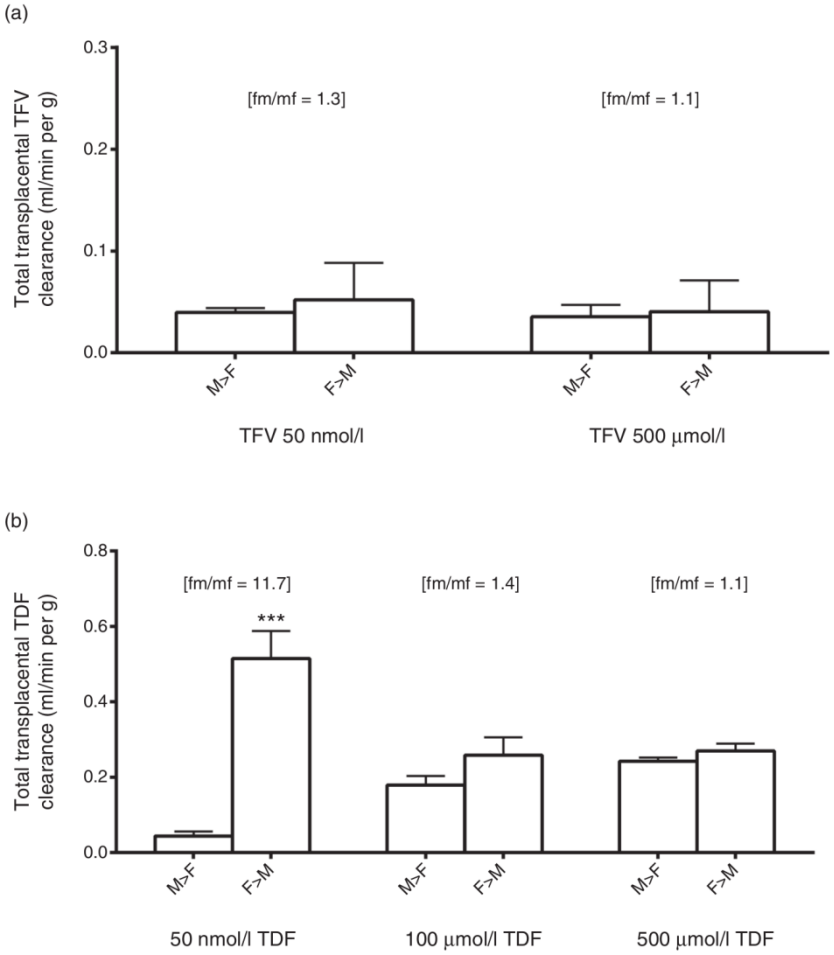

Fig. 1. Transport of TFV (a) and TDF (b) across the dually perfused rat term placenta in M>F and F>M direction. The tested compound was added to either maternal or fetal compartment and concentration was measured radiometrically in fetal venous outflow. Total transplacental clearance was calculated by eq. 1 and 2 (see Materials and Methods). No significant asymmetry either at low (50 nmol/l) or high (500 μmol/l) TFV concentration was detected. On the contrary, at low TDF concentration, significant asymmetry in transplacental clearance was observed in favour of F>M direction. Increasing TDF concentration to 500 μmol/l annulled this asymmetry, confirming saturation of transporter(s). Numbers in brackets show the ratio between F>M and M>F clearances; data are presented as means ± SD of at least three experiments. Student's *t*-test was used to evaluate statistical significance, $^{***}P < 0.001$.

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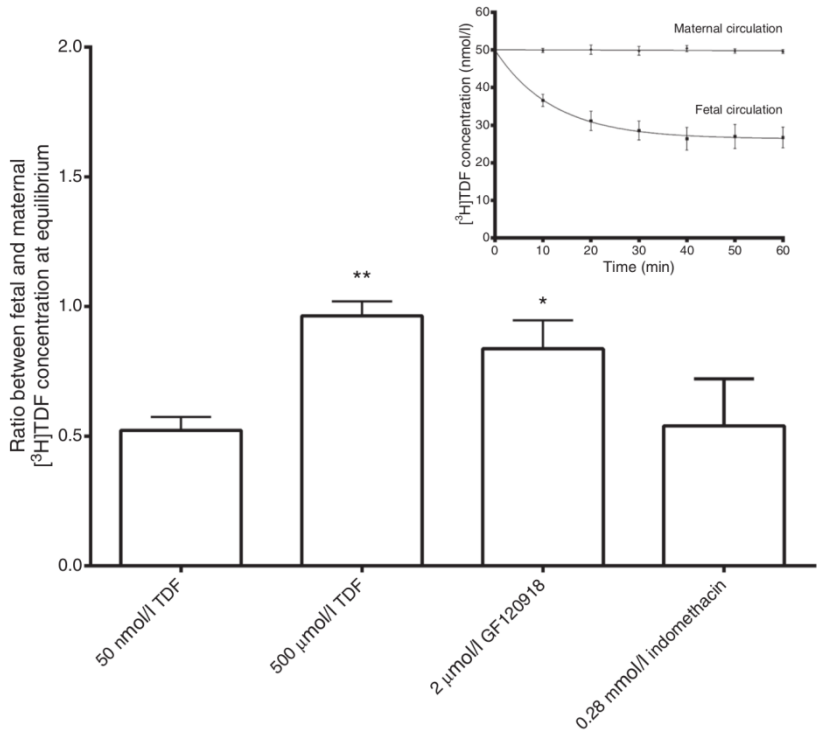

Fig. 2. Effect of inhibitors on elimination of TDF from the fetal circulation. In the closed-circuit perfusion setup, both fetal and maternal sides of the placenta were simultaneously infused with 50 nmol/l concentration of [^{3}H]TDF. Fetal perfusate was recirculated for 60 min and at the end of experiment, fetal and maternal concentrations of TDF were compared. Fetal [^{3}H]TDF concentration decreased from 50 nmol/l down to 25 nmol/l and stabilized after 40 min of perfusion (see inset). This decrease was significantly inhibited by cold TDF (500 μmol/l) and GF120918 (2 μmol/l). On the contrary, ABCC2 inhibitor indomethacin (0.28 mmol/l) had no effect on elimination of TDF from the fetal circulation. Data are presented as means ± SD of at least three experiments. $*P < 0.05$, $**P < 0.01$, statistically significant different from TDF 50 nmol/l (ANOVA followed by Bonferroni's test).

quantify their role in the transplacental pharmacokinetics of both compounds.

Using in-vitro transport experiments in MDCKII cells overexpressing human ABCB1, ABCG2 or ABCC2 we did not record any asymmetry in transepithelial translocation of TFV, indicating this compound is not a substrate of any of these transporters (Table 1). This correlates well with previously published data by Ray *et al.* [14] who used identical experimental model to exclude interactions between ABCB1 and TFV. We also support recent findings by Cihlar *et al.* [15] and Rodriguez-Novoa *et al.* [26] who suggested no role of ABCC2 in TFV pharmacokinetics. In addition, we provide the first evidence that ABCG2 does not mediate TFV transport.

TDF, on the contrary, showed strikingly different behaviour in comparison with TFV. When investigating its transport across ABCB1-transduced cells in basal-to-apical vs. apical-to-basal direction, TDF achieved transport ratio (r) 2–2.6 times higher than that observed in the parental cell line. Furthermore, application of GF120918 (2 μmol/l) abolished this asymmetry, resulting in translocation of TDF across the cell monolayer by mechanism of passive diffusion ($r \approx 1$). These findings clearly confirm TDF as a substrate of ABCB1 transporter as suggested recently [17,18]. Based on our findings, TDF seems to be a substrate as strong as colchicine, model substrate often used for in-vitro assays [27], topotecan [22] or other antiretrovirals including zidovudine [28], abacavir [29] or lopinavir [30].

Similarly, in-vitro studies in ABCG2-transduced MDCKII cells revealed that TDF is transported by ABCG2. To our knowledge we provide the first evidence that pharmacokinetics of TDF can be affected by ABCG2 transporter. Conversely, Janneh *et al.* have recently reported [31] that application of dipyridamole, a nonspecific inhibitor of ABCB1 and ABCG2, resulted in insignificant increase in TDF accumulation within peripheral mononuclear cells suggesting that TDF is not a substrate of ABCG2. However, since their method failed to reveal transport of TDF by ABCB1 either, we speculate that low sensitivity of the experimental approach prevented disclosure of interaction between TDF and both ABC transporters in their study.

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To confirm our in-vitro findings on the organ level, we employed the method of dually perfused rat term placenta, a well established method to study placental pharmacology [23,24,32,33] and physiology [34,35]. As both rat and human placenta express abundant amounts of ABCB1 [9] and ABCG2 [10], we introduced this model as a suitable tool to investigate the role of placental ABCB1 [33] and ABCG2 [23] in placental disposition of drugs. In open-circuit perfusion setup, clearances of TFV in both fetal-to-maternal and maternal-to-fetal directions were comparable and independent of drug concentration. These findings indicate linear pharmacokinetics without involvement of transporter-mediated mechanism(s) (Fig. 1) and correspond well with our results obtained *in vitro*. Furthermore, low values of TFV transplacental clearances suggest its restricted passage across the placenta when compared with antipyrine, a marker of passive diffusion [24]. This is in accordance with observations by Nirogi *et al.* [36] who found TFV in the placenta but not in amniotic fluid and fetal tissues when administered as a single fixed dose of efavirenz-emtricitabine-TDF in rats. Poor TFV transport was also observed in other biological membranes such as the intestine [37], blood–brain barrier [38] or blood–cerebrospinal barrier [39] most likely due to physical–chemical properties of the molecule, that is its anionic charge at physiological pH and low lipid-solubility of the nonionized fraction. Therefore, it is reasonable to assume that this molecule cannot readily cross cell membranes by passive diffusion; however, involvement of other transporters, such as equilibrative nucleoside transporters and/or concentrative nucleoside transporters, in TFV pharmacokinetics cannot be excluded.

Once absorbed, TDF is cleaved by nonspecific esterases, thus occurring in the systemic circulation predominantly as TFV [40]. However, esterase-mediated degradation of TDF can be inhibited by concomitantly administered treatment or substances normally present in nutrition such as fruit esters [17,18]. It can be hypothesized that TDF, at least to some extent, can also circulate in the maternal blood and reach the placental barrier; therefore, in this study we investigated placental transfer of TDF as well. In contrast to TFV, we observed great asymmetry in TDF transplacental clearances between mother to fetus and fetus to mother. In detail, transport from fetus to mother was 11.7 times faster than that in the opposite direction. In addition, placental transport of this drug was concentration-dependent in both directions indicating involvement of a saturable transport mechanism. Furthermore, employing closed-circuit perfusion setup with low, nonsaturating TDF concentrations, we observed transport of the drug from fetal to maternal circulation against concentration gradient confirming active transport mechanism. This transport was significantly reduced by administration of GF120908, a common ABCG2 and ABCB1 inhibitor, confirming ability of these transporters to mediate passage of TDF from fetus to mother (Fig. 2). Taking these results together with findings from in-vitro transport experiments in MDCKII-transduced cells, we conclude that both ABCB1 and ABCG2 affect TDF transplacental kinetics. On the contrary, indomethacin, a nonspecific inhibitor of ABCC(s), did not affect TDF transplacental transport, indicating that ABCC2 does not modulate transplacental passage of TDF. As drug–drug interactions on ABC transporters may substantially affect the fate of drugs in organism, our findings should be taken into account when TDF is co-administered with compounds whose membrane transport is mediated by ABCB1 and/or ABCG2 such as antiretrovirals [41] or other drugs administered in pregnancy [8]. Differences in ABCB1 expression between HIV-infected and noninfected women [42] as well as genetic polymorphisms leading to altered expression and function of the protein in the placenta [43] should also be considered when substrate drugs are prescribed.

In summary, our cell-based results suggest that TFV does not interact with human ABCB1, ABCG2 or ABCC2 transporters, whereas TDF was shown to be a dual substrate of both ABCB1 and ABCG2 but not of ABCC2. Our in-situ experiments in perfused rat placenta confirmed these findings, suggesting that ABCB1 and ABCG2, but not ABCC2, play an important role in efflux of TDF from fetus to mother. We propose limited mother-to-fetus transport of both TFV and TDF. While placental transport of TFV is restricted passively, by physical–chemical properties of the molecule, limited mother-to-fetus passage of TDF is actively mediated by placental ABCB1 and ABCG2 transporters, pumping this compound from trophoblast back to maternal circulation.

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Z.N. performed in-situ placenta perfusion experiments, analysed the data and participated in writing the manuscript; L.C. designed and performed in-vitro cell-based experiments, analysed the data and participated in writing the manuscript; M.C. cultivated the cells, designed and performed in-vitro cell-based experiments and critically revised the article; F.S. designed and supervised the experiments, analysed the data and participated in writing the article.

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Conflicts of interest

There are no conflicts of interest.

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7.2 Effect of drug efflux transporters on placental transport of antiretroviral agent abacavir

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Tato práce, která popisuje interakce antiretrovirotika abakaviru s hlavními ABC transportéry v placentě, vznikla na základě spolupráce s Univerzitou v Manchesteru. Položili jsme si v ní za cíl objasnit, zda je abakavir substrátem ABCB1, ABCG2, ABCC2 a ABCC5 a následně zhodnotit úlohu ABC transportérů v transplacentární farmakokinetice tohoto často používaného léčiva u těhotných HIV pozitivních žen.

Za použití *in vitro* metody využívající transportní experimenty na MDCKII buňkách transdukovaných lidskými transportéry jsme zjistili, že abakavir je substrátem ABCB1 i ABCG2, ale neinteraguje s ABCC2 ani s ABCC5. Během *in situ* experimentů na duálně perfundované potkaní placentě jsme detekovali vysoký transplacentární přestup abakaviru v obou směrech bez známek aktivního transportu. Při použití uzavřeného perfuzního systému, kdy je minimalizován vliv pasivní difúze, jsme však pozorovali aktivní eflux abakaviru z fetálního kompartmentu, který byl zprostředkován transportéry ABCB1 a ABCG2. *Ex vivo* akumulační studie na lidských placentárních fragmentech neprokázaly, že by zmíněné transportéry u člověka ovlivňovaly uptake abakaviru placentou.

Závěrem lze říci, že abakavir interaguje s ABCB1 a ABCG2. Nicméně na transplacentárním přestupu abakaviru se uplatňuje i vliv pasivní difúze a/nebo účast jiných transportérů, které mohou jeho interakce s ABC transportéry maskovat.

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Effect of drug efflux transporters on placental transport of antiretroviral agent abacavir

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ABSTRACT

Abacavir is as a frequent part of combination antiretroviral therapy used in pregnant women. The aim of this study was to investigate, using *in vitro*, *in situ* and *ex vivo* experimental approaches, whether the transplacental pharmacokinetics of abacavir is affected by ATP-binding cassette (ABC) efflux transporters functionally expressed in the placenta: P-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2), multidrug resistance-associated protein 2 (ABCC2) and multidrug resistance-associated protein 5 (ABCC5). *In vitro* transport assays revealed that abacavir is a substrate of human ABCB1 and ABCG2 transporters but not of ABCC2 or ABCC5. In addition, *in situ* experiments using dually perfused rat term placenta confirmed interactions of abacavir with placental Abcb1/Abcg2. In contrast, uptake studies in human placental villous fragments did not reveal any interaction of abacavir with efflux transporters suggesting a large contribution of passive diffusion and/or influx mechanisms to net transplacental abacavir transfer.

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1. Introduction

According to the World Health Organization, all HIV infected pregnant women are eligible for antiretroviral treatment to prevent transmission of the virus to their children. Combination antiretroviral therapy (cART) can significantly reduce the number of infected infants [1], and therefore recent guidelines have recommended that chronic cART should be initiated in HIV positive pregnant woman as soon as possible [2,3].

It is widely accepted that significant reduction in mother-to-child-transmission (MTCT) of HIV in pregnant women receiving antiretroviral treatment is primarily caused by reduced maternal viral load in blood and genital secretions [3]. However, even among women with extremely low HIV RNA levels, cART has been shown to reduce the risk of transmission [4]. This suggests that an additional prophylactic mechanism may be involved when the antiretroviral drug crosses the placenta and reaches systemic antiretroviral levels in the fetus [3]. It is crucial to ensure adequate antiretroviral exposure in the fetus, while minimizing drug-associated toxicities, in the care of pregnant HIV-infected women. Therefore, detailed knowledge of all placental factors potentially affecting drug disposition of the fetus is important for rational selection of safe and effective cART in pregnancy.

In general, the transplacental exchange of drugs occurs via passive diffusion or is transporter-mediated [5]. Among placental drug transporters, ATP-binding cassette (ABC) drug efflux transporters, namely P-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2) and multidrug resistance-associated proteins (ABCCs), have been shown to actively transport their substrates from fetus to mother, and it has been well documented that these ABC transporters may affect the transplacental pharmacokinetics of many clinically used drugs, including antiretrovirals [6,7].

Abacavir, a nucleoside reverse transcriptase inhibitor, is very efficient in reducing the perinatal transmission of HIV [8] and, therefore, has recently been recommended as one of the preferred antiretrovirals for pregnant women [2]. Limited data have so far suggested high maternal-to-fetal transport of abacavir at the time of delivery [9,10], probably via a mechanism of passive diffusion

Abbreviations: ABC, transporters; ATP, -binding cassette transporters; ABCG2, breast cancer resistance protein; ABCB1, P-glycoprotein; ABCC, multidrug resistance-associated protein; cART, combination antiretroviral therapy; MTCT, mother-to-child transmission.

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without involvement of a saturable transport system [11]. Some studies, however, have shown the potential of abacavir to interact with ABC transporters, such as ABCB1, ABCG2 [12–14] and ABCCs [15]. Nevertheless, the role of ABC transporters in the passage of abacavir across the placenta has not yet been fully elucidated.

The aim of the present study was to describe interactions of abacavir with selected ABC transporters by employing *in vitro* transport assays across MDCKII monolayers overexpressing human ABCB1, ABCG2, ABCC2 or ABCC5. To detect whether these transporters affect transplacental passage on the organ level, we further investigated abacavir transport across dually perfused rat term placenta and measured abacavir uptake in the fresh villous fragments of human placenta.

2. Materials and methods

2.1. Reagents and chemicals

Abacavir used for the *in vitro* studies was kindly provided by the NIH AIDS Research and Reference Reagent program. Abacavir was dissolved in DMSO; concentration of DMSO in *in vitro* experiments did not exceed 0.05%. Radiolabeled abacavir ([^{3}H]-abacavir) and vinblastine ([^{3}H]-vinblastine), were purchased from Moravek Biochemicals (CA, USA). Dual ABCB1/ABCG2 inhibitor elacridar was kindly provided by GlaxoSmithKline (Greenford, UK), ABCB1 inhibitor zosuquidar was obtained from Toronto Research Chemicals (North York, ON, Canada). Indomethacin, a non-selective inhibitor of ABCC(s), was purchased from Sigma–Aldrich (St. Louis, MO). Pentobarbital (Nembutal) was obtained from Abbott Laboratories (Abbott Park, IL, USA). Bradford protein assay was purchased from BioRad (Richmond, CA). All other chemicals were analytical grade.

2.2. Cells

MDCKII (Madine-Darby Canine Kidney) parental cell line and MDCKII cells overexpressing ABCB1 (MDCKII–ABCB1), ABCG2 (MDCKII–ABCG2), ABCC2 (MDCKII–ABCC2) and ABCC5 (MDCKII–ABCC5) were provided by the Netherlands Cancer Institute (Dr. A. Schinkel) and cultured in DMEM complete high glucose medium with L-glutamine supplemented with 10% FBS.

2.3. Animals

Pregnant Wistar rats were purchased from MediTox Ltd. (Konarovice, Czech Republic) and maintained in 12/12 h day/night standard conditions with water and pellets *ad libitum*. Experiments were performed on day 21 of gestation. Fasted rats were anesthetized by administering a dose of 40 mg/kg pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL) into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

2.4. Transport experiments in vitro

MDCKII-parental and MDCKII cells overexpressing ABCB1, ABCG2, ABCC2 or ABCC5 were seeded at a density of 1.0×10^6 cells per well and cultured for 72 h to confluence with daily medium replacement. Transport assays were performed on microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, NY) for two sets of experimental conditions, as described below.

Bidirectional transport assays were used to quantify abacavir transport across cell monolayers in the apical-to-basolateral (AB) and basolateral-to-apical (BA) directions, as described in our previous studies [16,17]. One hour before starting the experiment, cells were washed with PBS (37 °C) and incubated with 2 ml of Opti-MEM (Invitrogen, Carslbad, CA) either alone or containing the dual ABCB1 and ABCG2 inhibitor elacridar (2 μM) [17]. The experiment was started by replacing the medium in the donor compartment (either apical or basolateral) with fresh Opti-MEM (37 °C) containing [^{3}H]-abacavir alone or with elacridar (2 μM). The final activity of abacavir was 0.04 μCi/ml and was tested at a low concentration of 200 nM as dictated by the specific activity of radioisotopes required for analysis. Abacavir bidirectional transport was also studied at 50 μM concentration as published previously [14]. Aliquots of 50 μl were collected at 2, 4 and 6 h from the acceptor compartment and radioactivity was measured by liquid scintillation counting (Tri-Carb 2900 TR PerkinElmer). The percentage of radioactivity in the acceptor compartment relative to the stock solution initially added to the donor compartment was calculated. Ratios of BA to AB translocation after 6 h incubation (r) were also calculated [16].

Concentration equilibrium transport assays were used to evaluate abacavir active transport akin to previously published studies [18,19], using the same cell cultures and seeding conditions as in the bidirectional transport assays. In these experiments, after 1 h preincubation period, the transport study was initiated by adding abacavir (200 nM or 50 μM) traced with [^{3}H]-abacavir (final activity 0.04 μCi/ml) with or without inhibitor elacridar (2 μM) to both (apical and basolateral) sides of the monolayer to give the same initial abacavir concentration in both compartments. Aliquots of 50 μl were collected at 2, 4 and 6 h from both compartments and radioactivity was measured by liquid scintillation counting. The percentage of radioactivity in the apical and basolateral compartments relative to the initial stock solution was calculated [19]. Ratios of the percentages reached after 6 h incubation (r) were calculated.

In both *in vitro* setups, leakage of FITC-dextran was analyzed at the end of the experiments and deemed acceptable if it was ≤ 1% per hour.

2.5. Dual perfusion of rat placenta

The method involving dually perfused rat term placenta was used as described previously [20]. In brief, one uterine horn was excised and submerged in heated Ringer's saline. A catheter was inserted into a uterine artery proximal to blood vessels supplying a selected placenta and then connected to a peristaltic pump. Krebs's perfusion liquid containing 1% dextran was supplied from the maternal reservoir at a rate of 1 ml/min. The uterine vein, including anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that the maternal solution could exit the perfused placenta. The selected fetus was separated from neighboring fetuses by ligatures. The umbilical artery was catheterized using a 24-gauge catheter connected to the fetal reservoir and perfused at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner and the selected fetus removed. Before the start of each experiment, the fetal vein effluent was collected in a preweighed glass vial to check for possible leakage of the perfusion solution from the placenta. If any leakage was detected, the experiment was terminated. Maternal and fetal perfusion pressures were maintained at levels close to physiological values and monitored continuously throughout the perfusion experiments as described previously. At the end of experiment, the placenta was perfused with radioactivity-free buffer for 10 min, then excised from the uterine tissue, dissolved in tissue solubilizer (Solvable; PerkinElmer Life and Analytical Sciences) and the radioactivity measured to detect tissue-bound abacavir.

Two types of perfusion systems were used:

Open-circuit perfusion system. This system was employed to study fetal-to-maternal (F→M) and maternal-to-fetal (M→F) clearances of abacavir. A trace amount of [^{3}H]-abacavir (300 nM) was added to either the maternal (M→F studies) or fetal (F→M studies) reservoir immediately after successful surgery. After a 5 min stabilization period, sample collection was started (time 0 min). Fetal effluent samples were placed in preweighed vials at 5 min intervals, the abacavir concentration was measured radiometrically and transplacental clearance was calculated at all measured intervals as described below.

Closed-circuit (recirculation) perfusion system. This system was employed to further study the involvement of placental transporter(s) on abacavir transplacental transport. Both the maternal and fetal sides of the placenta were infused with 300 nM [^{3}H]-abacavir. Elacridar, a dual inhibitor of ABCB1 and ABCG2, or indomethacin, a nonspecific inhibitor of ABCCs [17,21], were added to both maternal and fetal circulations in order to identify transporters responsible for abacavir active transplacental transport. After a short-time stabilization period, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (250 μl) were collected every 10 min from the maternal and fetal reservoirs, and concentration of [^{3}H]-abacavir measured. This experimental setup ensured a steady concentration on the maternal side of the placenta and enabled investigation of the fetal/maternal ratio; any net transfer of the substrate implied transport against a concentration gradient and provided evidence of active transport.

2.6. Pharmacokinetic analysis of efflux transport activity in rat placenta

The principle of organ clearance was applied to mathematically describe M→F and F→M transport of abacavir in the open-circuit perfusion system. Averaged data recorded at intervals of 10–35 min were used for the following calculations. The M→F transplacental clearance (Cl_{mf}) of abacavir normalized to placenta weight was calculated according to Eq. (1):

$$Cl_{mf} = \frac{C_{fv} \times Q_f}{C_{ma} \times W_p} \quad (1)$$

where C_{fv} is the concentration of abacavir in the umbilical vein effluent, Q_f is the umbilical flow rate, C_{ma} is the concentration of abacavir in the maternal reservoir and W_p is the wet weight of the placenta. The F→M transplacental clearance (Cl_{fm}) was calculated according to Eq. (2):

$$Cl_{fm} = \frac{(C_{fa} - C_{fv})\,Q_f}{C_{fa} \times W_p} \quad (2)$$

where C_{fa} is the concentration of abacavir in the fetal reservoir entering the perfused placenta via the umbilical artery.

2.7. Functional studies in fresh villous fragments from human placenta

Accumulation of [^{3}H]-abacavir and [^{3}H]-vinblastine (a model ABCB1 substrate) by placental villous fragments was measured in the presence/absence of dual ABCB1/ABCG2 inhibitor (2 μM elacridar) or ABCB1 inhibitor (1 μM zosuquidar) using a method as described previously [22]. Briefly, placentas were collected at term after uncomplicated pregnancies, from women at St. Mary's Hospital, Manchester following their written informed consent as approved by the Local Research Ethics Committee. Small fragments of villous tissue were dissected within 30 min of delivery. These were washed in Tyrode's solution (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM Mg Cl_2, 10 mM HEPES, 5.6 mM glucose, pH 7.4) and were then tied to hooks supported by perspex rods. After 10 min pre-incubation of fragments at 37 °C in Tyrode's with or without inhibitors, uptake kinetics of [^{3}H]-abacavir was initially measured at six time points (5, 15, 30, 60, 120 and 180 min). Based on these pilot experiments and the previous study [22] the uptake of [^{3}H]-abacavir and [^{3}H]-vinblastine in the presence or absence of inhibitors was compared at a timepoint of 180 min, chosen as optimal to recognize inhibitory effect of efflux transporters. All uptake experiments were made in triplicate. Incubations were carried out at 37 °C in Tyrode's solution containing [^{3}H]-vinblastine (0.2 μCi/ml, 8 nM) and [^{3}H]-abacavir (0.5 μCi/ml, 5 μM) either with or without 2 μM elacridar or 1 μM zosuquidar. After incubation, fragments were washed (2 × 15 s) in ice cold Tyrode's and then lysed in distilled water to release accumulated isotope. The lysates were counted using a liquid scintillation β-counter (Tri-Carb 2100TR; Packard Bioscience) to quantify [^{3}H] activity. The fragments were then dissolved in 0.3 M NaOH and aliquots assayed for protein using a Bio-Rad protein assay. Accumulation of the tested substrates was calculated as pmol/mg protein. Abacavir concentration used in these studies was 0.5 μCi/ml (5 μM) which corresponds with abacavir plasma concentrations detected in pregnant women (approximately 7 μM) [10].

2.8. Statistical analysis

For the *in vitro* cell-based studies, the two-sided unpaired Student's *t*-test was employed to evaluate statistical significance. In the *in situ* placenta perfusion studies and placental fragment tissues study, statistical significance was examined by either two-sided unpaired Student's *t*-test or one way ANOVA followed by Bonferroni's test. All data were assessed using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Abacavir transport across cell monolayers—bidirectional transport assays

We first determined the transepithelial transport of [^{3}H]-abacavir at 200 nM concentration through monolayers of parental and ABCB1-, ABCG2-, ABCC2- and ABCC5-overexpressing cells. No asymmetry in BA *vs.* AB transport of abacavir was observed in MDCKII-parental cells and cells transduced with ABCC2 and ABCC5 ($r \approx 1$). In contrast, significantly larger BA/AB ratios were observed in ABCB1 and ABCG2 overexpressing cells ($r = 3.24$ and 2.81, respectively). Increasing the abacavir concentration to 50 μM had no significant effect on abacavir transport in the latter cells ($r = 3.44$ and 2.88, respectively). Addition of a dual ABCB1 and ABCG2 inhibitor, elacridar (2 μM), completely abolished the asymmetry in the translocation of abacavir in the respective cell lines, yielding transport ratios of approximately one. These findings suggest that abacavir is a substrate of human transporters ABCB1, ABCG2 but not ABCC2 or ABCC5 (Table 1).

3.2. Abacavir transport across cell monolayers—concentration equilibrium transport assays

As abacavir is a very lipophilic compound (log P 1.2) [23], we used highly sensitive concentration equilibrium transport assays [19] to confirm the results obtained from the bidirectional transport assays. Under concentration equilibrium conditions, increased abacavir concentration in the apical compartment and decreased abacavir concentration in the basolateral compartment was observed after 6 h incubation in MDCKII–ABCB1 ($r = 2.20$) and MDCKII–ABCG2 ($r = 2.03$). When abacavir was tested at a higher concentration (50 μM), active abacavir transport across the cells was detected, with comparable ratios in MDCKII–ABCB1 ($r = 2.49$)

Table 1
Abacavir transport in MDCKII parental and ABCB1-, ABCG2-, ABCC2- and ABCC5-overexpressing cells after 6 h incubation using bidirectional transport assays.

Cell line (MDCKII)	Drug concentration (with or without inhibitor)	Transport ratio (BA/AB)
Parental	200 nM	1.00 ± 0.06
ABCB1	200 nM	3.24 ± 0.13***
	200 nM + elacridar (2 μM)	1.03 ± 0.02
	50 μM	3.44 ± 0.03***
	50 μM + elacridar (2 μM)	1.01 ± 0.01
ABCG2	200 nM	2.81 ± 0.27***
	200 nM + elacridar (2 μM)	1.12 ± 0.14
	50 μM	2.88 ± 0.16***
	50 μM + elacridar (2 μM)	1.05 ± 0.09
ABCC2	200 nM	1.00 ± 0.05
ABCC5	200 nM	1.05 ± 0.04

Data points are means ($n = 3$) ± SD, Student's t-test was employed to compare AB (apical-to-basolateral) and BA (basolateral-to-apical) translocation.
ABCB1, *P*-glycoprotein; ABCG2, breast cancer resistance protein; ABCC2, multidrug resistance-associated protein 2; ABCC5, multidrug resistance-associated protein 5.
*** $p < 0.001$.

and MDCKII–ABCG2 ($r = 1.92$). Use of the dual ABCB1/ABCG2 inhibitor elacridar (2 μM) completely blocked active abacavir transport (both ratios ≈1), confirming that ABCB1 and ABCG2 play a role in abacavir transcellular transport (Fig 1). Similarly, in MDCKII-parental cells and cells overexpressing ABCC2 and ABCC5, no change in abacavir concentration was observed in either the apical or basolateral compartments ($r \approx 1$), suggesting that no active transporters are involved in abacavir transport across these cells.

3.3. Open circuit placenta perfusion experiments: detection of transplacental clearances in the M → F and F → M directions

When the maternal or fetal side of the placenta was infused with abacavir at 300 nM concentration, the transplacental clearances were comparable in both directions, indicating no significant transporter-mediated abacavir efflux transport (Fig. 2). Less than 1% of the abacavir dose was detected in the placenta after the perfusion experiments, suggesting limited tissue binding and negligible effect on the clearance calculation.

3.4. Closed circuit placenta perfusion experiments: effect of inhibitors on abacavir transport across the placenta

To further study abacavir transplacental transport, both sides of the placenta were perfused with the same concentration of [^{3}H]-abacavir (300 nM) in a closed circuit experimental setup. We observed a slight but significant decrease of abacavir concentration in the fetal perfusate, suggesting active transport against the concentration gradient from the fetal to maternal side of the placenta. This decline was significantly blocked by coinfusion with either elacridar (2 μM, dual inhibitor of ABCB1 and ABCG2) or indomethacin (0.28 mM, nonspecific inhibitor of ABCCs) (Fig. 2). These results indicate that abacavir transplacental transport is affected by the activity of placental ABC drug efflux transporters.

3.5. ABCB1 and ABCG2 activity in fresh placental fragments; effect on uptake of abacavir

The role of ABCB1 and ABCG2 transporters in efflux of abacavir from the human placenta villous tissue was examined by uptake assay using elacridar and zosuquidar as inhibitors. The inhibitory effect of elacridar and zosuquidar in this experimental setup has been confirmed by 1.65 and 1.76 fold increase, respectively, in [^{3}H]-vinblastine uptake at 180 min timepoint ($p < 0.01$). No increase

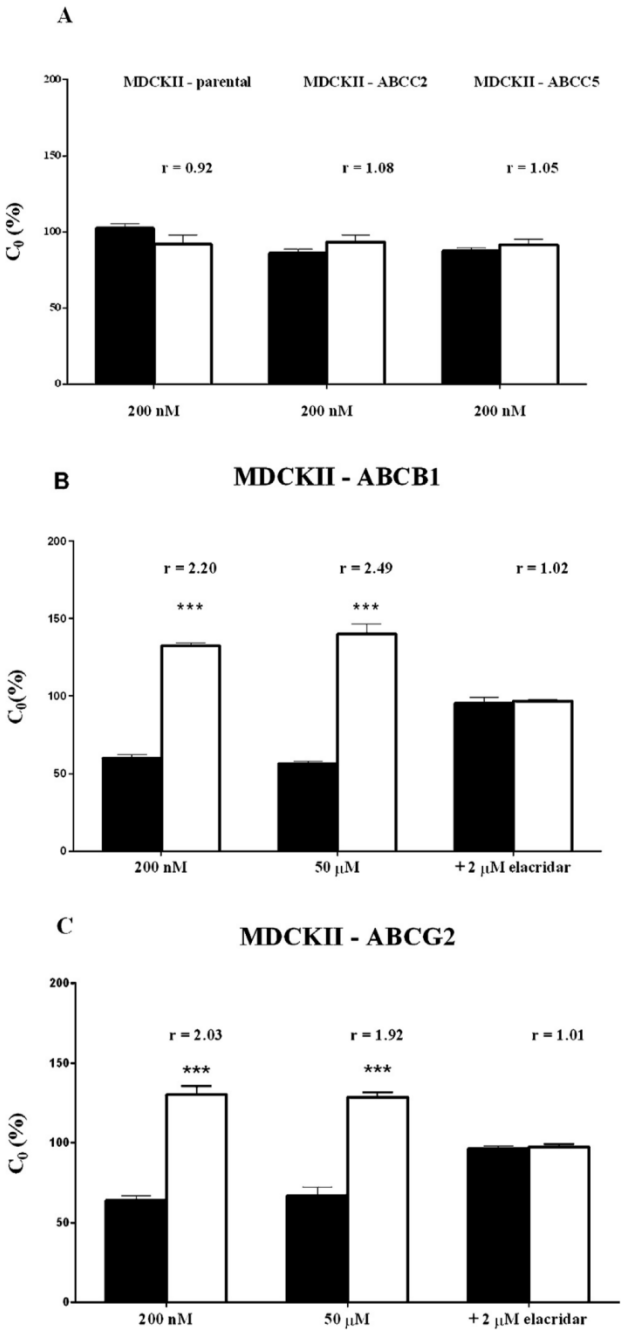

Fig. 1. Abacavir transport in MDCKII parental and ABCB1-, ABCG2-, ABCC2- and ABCC5-overexpressing cells after 6 h incubation under concentration equilibrium conditions. Percentage of initial concentration (C_0) obtained in the basolateral (black columns) or apical compartment (white columns) after 6 h incubation measured by concentration equilibrium assay using MDCKII parental and MDCKII cells overexpressing ABCC2 and ABCC5 (A), ABCB1 (B) and ABCG2 (C) at various concentrations, with or without inhibitor. Ratio (r) between concentrations in the apical and basolateral compartments measured at the end of experiment. Data points are means ± SD, $n = 3$, Student's t-test was employed to compare abacavir concentrations at the end of experiment, ***$p < 0.001$.

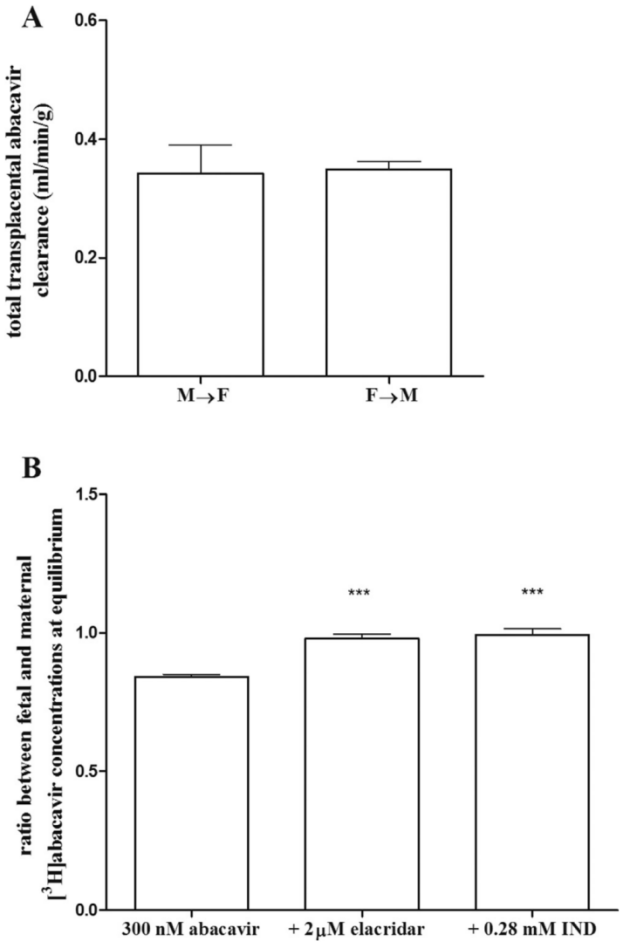

Fig. 2. Transport of abacavir across dually perfused rat term placenta in the open (A) and closed (B) experimental setup. [^{3}H]-abacavir (300 nM) was added to either the maternal or fetal compartment and [^{3}H]-abacavir concentration was measured radiometrically in fetal venous outflow. Total transplacental M → F and F → M clearances were calculated by Eqs. (1) and (2) (see Section 2), respectively. In the closed-circuit perfusion setup, both fetal and maternal sides of the placenta were simultaneously infused with 300 nM [^{3}H]-abacavir. Fetal perfusate was recirculated for 60 min and at the end of experiment, fetal and maternal concentrations of abacavir were compared. A slight but significant decrease of abacavir concentration in the fetal perfusate was observed. This decrease was significantly inhibited by elacridar (2 μM) and indomethacin (IND 0.28 mM). Data are presented as means ± SD, $n \geq 3$, $^{***}p < 0.001$ statistically significant different from abacavir 300 nM (ANOVA followed by Bonferroni's test).

in accumulation of [^{3}H]-abacavir was observed when elacridar or zosuquidar inhibitors were present (Fig. 3).

4. Discussion

Although current antiretroviral therapy effectively reduces the risk of vertical transmission of HIV, its use may have harmful effects on the fetus. To fully assess the safety profile of antiretroviral treatment in pregnant women, it is important to describe all factors potentially affecting transplacental pharmacokinetics, such as interactions with ABC drug efflux transporters localized in the placenta [24–28]. In our previous study, we used a combination of *in vitro* and *in situ* experimental approaches to describe interactions of tenofovir and its prodrug, tenofovir disoproxil fumarate, with placental drug efflux transporters and quantified their role in transplacental pharmacokinetics [17]. Abacavir has recently been classified as one of the preferred antiretrovirals for the prevention of MTCT of HIV in the United States [3], however, data evaluating abacavir use in pregnancy are still very limited.

Regarding abacavir transplacental transport, the ratio between abacavir concentrations in maternal and cord blood after delivery in a limited number of mothers and their infants has previously been detected as ~1, suggesting high maternal-to-fetal transport [9,10]. Using a human placental transfer model, abacavir transport has also been shown to be rapid and likely mediated by passive diffusion [11], in accordance with the high lipid solubility of the molecule. However, abacavir transcellular transport has also been shown to be influenced by activity of membrane transporters [12,13,29,30]. Nevertheless, the effect of these transporters on abacavir passage across the placenta has not yet been elucidated.

Shaik et al. [12] identified abacavir as a potential substrate of human ABCB1 transporter using several *in vitro* methods, including ATPase assay and accumulation and transport studies with MDCKII cells overexpressing human ABCB1. In the case of ABCG2, abacavir has been reported to affect the ABCG2-mediated transport of pheophorbide A [15]. MDCKII cells transfected with murine ortholog of ABCG2 exhibited polarized transport of abacavir [30]. Moreover, deletion of both Abcb1 and Abcg2 was shown to significantly affect brain abacavir distribution in an *in vivo* mouse model, confirming the important role of these transporters in abacavir pharmacokinetics [12,13].

Using MDCKII cell line, a well-established model for detection of ABC transporter substrates [31], we revealed abacavir BA/AB transport ratios $r > 2$ at both concentrations tested in both ABCB1 and ABCG2 cells. We did not observe any tendency to saturation confirming findings of Pan et al. [14]. It is widely accepted that BA/AB transport ratio exceeding $r = 2$ is an indicator of ABC transporter

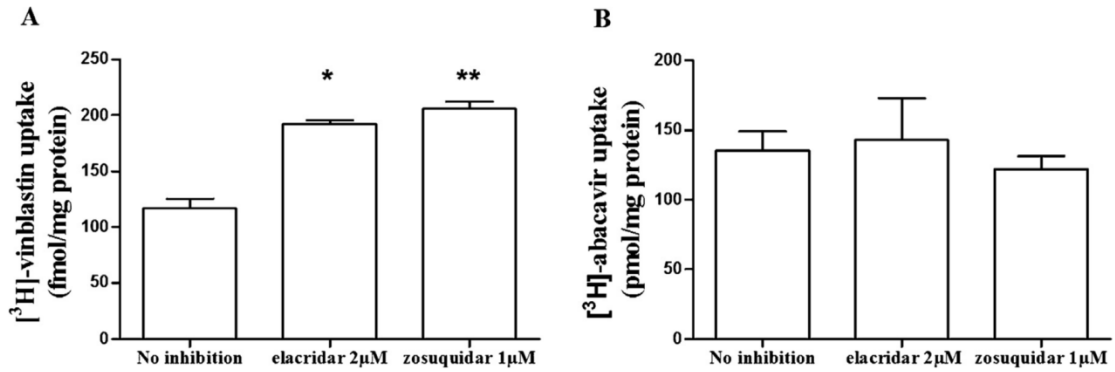

Fig. 3. Accumulation of [^{3}H]-vinblastine (A) and [^{3}H]-abacavir (B) in the presence and absence of 2 μM elacridar and 1 μM zosuquidar in fresh human placental fragments after 180 min of incubation. Elacridar and zosuquidar significantly increased accumulation of vinblastine ($^*p < 0.05$; $^{**}p < 0.01$, one-way ANOVA), while no effect of the inhibitors was observed on the accumulation of abacavir. Data are mean ± SD, $n = 3$ placentas.

substrates in MDCKII cells [31]; we therefore assume that the interactions of abacavir with ABCB1 and ABCG2 transporters might be of *in vivo* importance. To the best of our knowledge we provide the first evidence that abacavir is a substrate of human ABCG2. Conversely, we detected no influence of human ABCC2 and ABCC5.

It has been demonstrated that conventional bidirectional transport assays fail to detect interactions of highly permeable compounds with ABCB1 [19], whereas more sensitive concentration equilibrium transport assays reveal active translocation of these compounds mediated by the same transporter. Therefore, we measured abacavir transepithelial transport under concentration equilibrium conditions in order to verify interactions with ABC transporters. As expected, abacavir was rapidly transported, even against concentration gradient, by ABCB1 and ABCG2 and this active transport was completely inhibited in the presence of elacridar, confirming our results obtained in the bidirectional transport assays. Nevertheless, neither ABCC2 nor ABCC5 were shown to affect abacavir membrane translocation.

To investigate the possible role of ABC transporters in abacavir transplacental pharmacokinetics *in situ* on the organ level, the well-established method of dually perfused rat term placenta was employed [17,20,32]. Using an open-circuit experimental setup, we detected comparable transplacental clearances in both directions. Employing an uptake assay in human placental fresh villous fragments, we did not see any effect of either elacridar or zosuquidar on accumulation of abacavir. These findings agree with data obtained from *ex vivo* perfusion studies using human placentas, which showed that abacavir had the highest clearance index of all tested antiretrovirals [33], supporting the hypothesis that abacavir is readily transferred across the placenta by passive diffusion. Interestingly, with a closed-circuit experimental setup, in which both sides of the rat placenta are supplied with equal abacavir concentrations, we observed significant decrease in abacavir concentration in the fetal compartment after 1 h perfusion. These findings suggest that abacavir can cross the placenta in the fetal-to-maternal direction against a concentration gradient. This transport was completely abolished in the presence of elacridar, confirming the involvement of ABCB1 and/or ABCG2.

The lack of interaction of abacavir with ABCB1 and ABCG2 transporters in the open-circuit experimental setup of rat placenta perfusion as well as in human placenta villous fragments could be explained by involvement of passive diffusion that lowers the effect of active transporters [34] or by concentrative/uptake transporters mediating transport abacavir across the placental membranes, *e.g.*, nucleoside transporters CNTs and ENTs (our unpublished data) whose functional expression has recently been confirmed in both, human and rat placentas [35,36]. We therefore hypothesize that under the conditions of open-circuit setup, in which the drug is presented from one side of trophoblast layer only, the uptake and/or equilibrative transporters might mediate transplacental transfer of abacavir to the trophoblast layer and thereby hinder the efflux function of ABCB1/ABCG2 transporters in these experimental conditions. In contrast, in the closed perfusion setup in which a concentration equilibrium on both sides of the placenta exists, the activity of transport mechanisms dependent on concentration gradient is reduced thus enabling detection of active ABCB1- and ABCG2-mediated efflux from the trophoblast cells.

Despite the fact that *in vitro* abacavir transport was not affected by human ABCC2 or ABCC5 transporters, when using the *in situ* rat placenta perfusion model, we observed a significant effect of the nonspecific ABCC inhibitor indomethacin on abacavir transplacental transport. We propose that interspecies differences, as well as involvement of other transporters potentially affected by indomethacin inhibition [37,38], may be responsible for this phenomenon.

Abacavir is extensively metabolized in the liver by uridine diphosphate glucuronyltransferase and alcohol dehydrogenase [39]. The influence of these enzymes on abacavir metabolism in the placenta in our experimental setups, however, seems to be unlikely as glucuronidation capacity of this organ is minimal [40] and the expression of placental alcohol dehydrogenase is negligible [41]. Regarding metabolism of abacavir in MDCKII cell line, Pan et al. [14] detected similar transport profiles for both radioactive and unlabeled compound (analyzed by HPLC method) thus ruling out possible contamination of transport experiments by cellular metabolism. Taken together, we believe that abacavir metabolism did not play any role in our experiments and did not affect our conclusions.

Besides potential drug–drug interactions, genetic polymorphisms of ABC transporters should also be considered when assessing fetal drug exposure to abacavir. Moreover, placenta is a dynamic organ, which undergoes considerable changes during gestation encompassing altered expression and function of placental transporters throughout pregnancy [42]. It can be therefore assumed that fetal exposure to abacavir may vary during gestation as well as across populations. In a recent study, significant associations between gestational age and concentrations of several antiretroviral drugs in fetal meconium have been observed, suggesting increasing exposure of the fetus from second trimester onwards [43]. It can be speculated that activity of several transporters might be more pronounced in earlier phases of gestation. Under such circumstances our findings might be of particular importance especially in cases when abacavir is combined with other substrates of ABCB1 or ABCG2 [44]. However, more data are required in order to establish individualized combination therapies for pregnant women.

In summary, we have demonstrated interactions of abacavir with the main placental drug efflux transporters. We propose that abacavir is a substrate for human ABCB1 and ABCG2, whereas ABCC2 and ABCC5 have no effect on abacavir transport. We also suggest high transfer of this compound across the placenta that can, nevertheless, be affected by the activity of ABCB1 and ABCG2. These findings should be considered in order to select safe and effective antiretroviral strategies for the prevention of perinatal transmission of HIV.

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Z.N. performed the *in situ* placenta perfusion experiments and analyzed the data, participated in the *in vitro* cell-based experiments and in the human placental fragment experiments and wrote the manuscript; L.C. designed and performed the *in vitro* cell-based experiments, analyzed the *in vitro* data and participated in writing the manuscript; S.G designed and supervised the experiments using human placental fragments and corrected the manuscript. M.C. performed the uptake study on human placental fragments, analyzed the data and assisted with writing the manuscript F.S. supervised the experiments, assisted with writing the manuscript and secured funding for the project.

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7.3 Role of ABCB1, ABCG2, ABCC2 and ABCC5 transporters in placental passage of zidovudine

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V této studii jsme se zabývali transplacentární farmakokinetikou AZT jako nejvýznamnějšího antiretrovirotika používaného u těhotných HIV pozitivních žen k léčbě a prevenci HIV infekce. Dali jsme si za cíl objasnit, zda je AZT substrátem ABCB1, ABCG2, ABCC2 a ABCC5 a zhodnotit zda jsou tyto transportéry schopny limitovat AZT v jeho transplacentárním přestupu z matky do plodu.

In vitro experimenty využívající buněčné transportní experimenty na MDCKII buňkách prokázaly, že AZT je substrátem ABCB1a ABCG2, zatímco ABCC2 ani ABCC5 neměly na transport AZT vliv. Vysoké a srovnatelné hodnoty transplacentární clearance napovídají, že AZT prochází přes placentu v obou směrech přibližně ve stejné míře bez vlivu aktivního transportu. Zapojením uzavřeného perfuzního systému, kdy byl efekt pasivní difúze na transport AZT minimální, jsme již byli schopni detekovat vliv ABCB1 a ABCG2, kteří aktivně odčerpávaly AZT z fetálního kompartmentu. Zajímavé je, že lamivudin, léčivo často podávané s AZT ve formě fixní kombinace, neovlivňovalo pozorované interakce AZT s ABCB1 ani s ABCG2 a to *in vitro* ani *in situ*.

Závěrem lze konstatovat, že AZT je substrátem ABCB1 a ABCG2, nicméně přestup AZT přes placentu není těmito transportéry limitován. Lamivudin a AZT spolu na testovaných transportérech neinteragovaly.

Role of ABCB1, ABCG2, ABCC2 and ABCC5 transporters in placental passage of zidovudine

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Running page title: role of ABC transporters in zidovudine transfer across the placenta

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Abstract

Zidovudine (AZT) is one of the most frequently used antiretroviral drugs in prevention of perinatal transmission of HIV. However, safety concerns on AZT use in pregnancy still persist as severe side effects are associated with AZT exposure in children. In our study we aimed to contribute to current knowledge on AZT transplacental transport and to evaluate potential involvement of the main human drug efflux ATP-binding cassette (ABC) transporters, p-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2) and multidrug resistance-associated proteins 2 and 5 (ABCC2 and ABCC5) in the disposition of AZT between mother and fetus. In order to elucidate this issue we investigated the effect of selected ABC transporters on AZT transepithelial transport across MDCKII cell monolayers. In addition we used the *in situ* method of dually perfused rat term placenta to further study the role of ABC transporters in AZT transplacental transport. *In vitro* studies revealed significant effect of ABCB1 and ABCG2 on AZT transport which was subsequently confirmed also on organ level. Lamivudine, an antiretroviral agent commonly co-administered with AZT, did not affect ABC transporter-mediated AZT transfer.

Keywords: zidovudine, p-glycoprotein, breast cancer resistance protein, multidrug resistance-associated protein 2, multidrug resistance-associated protein 5

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Introduction

Mother-to-child-transmission of HIV is the most common source of this infection in children occurring in 20 - 45 % of neonates born to HIV positive mothers [1]. In 1994 the first clinical trial focused on antiretroviral prophylaxis in pregnancy revealed that zidovudine (AZT), a nucleoside reverse transcriptase inhibitor (NRTI), could reduce the risk of vertical transmission by 67 % [2]. Since then this antiretroviral agent has become the gold standard in the care of pregnant HIV infected women frequently prescribed for antiretroviral combination therapy. Current medical interventions including appropriate antiretroviral therapy reduce perinatal transmission under 1 % [3]. However, even after 20 years in clinical use, safety concerns on AZT administration in pregnancy persist as numerous adverse effects have been reported in children exposed to AZT *in utero* [4-6], including recently reported heart anomalies [7]. Since certain adverse effects occurring in children have been described as concentration-dependent [8], to guarantee adequate and safe AZT-based therapy it is of particular importance to understand all factors potentially affecting transplacental transport of AZT.

Transplacental kinetics of drugs is frequently mediated by activity of membrane embedded ATP-binding cassette (ABC) efflux transporters [9]. Of these, p-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2) and multidrug-resistance-associated protein 2 and 5 (ABCC2, ABCC5) are the best described placental transporters affecting passage of their substrates into the fetal circulation [10-13].

Regarding the transplacental passage, AZT is suggested to readily cross the placenta from mother to fetus at the time of delivery [14-16] as well as during the first trimester [17] probably by the mechanism of passive diffusion [18-20]. However, evidence from *in vitro* and *in vivo* testing indicates that AZT transport across the cell membrane might be influenced

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by the activity of drug efflux ABC transporters [21-29]. Therefore, involvement of these transporters in AZT transplacental pharmacokinetics cannot be fully ruled out and has not been investigated satisfactorily so far.

As studies on interactions of AZT with ABC transporters have not been performed thoroughly and provide contradictory results [24, 25], we aimed to elucidate whether ABCB1, ABCG2, ABCC2 and/or ABCC5 recognize AZT as a substrate. Using *in vitro* transport assays in MDCKII cell lines and *in situ* dually perfused rat term placenta, we evaluated possible role of these transporters in AZT transplacental passage. Additionally, drug-drug interactions in placental transport between AZT and lamivudine, a commonly co-administered NRTI and suggested substrate of ABC transporters [23, 24, 30, 31], were investigated.

Material and methods

Reagents and chemicals

AZT and lamivudine were obtained from the NIH AIDS Research and Reference Reagent program and from Sigma-Aldrich (St. Louis, MO). Radiolabeled AZT ([^{3}H]-AZT) was purchased from Moravek Biochemicals (CA, USA). Dual ABCB1/ABCG2 inhibitor elacridar (GF120918) was kindly provided by GlaxoSmithKline (Greenford, UK). Indomethacin (IND), a non-selective inhibitor of ABCC(s), was purchased from Sigma-Aldrich. Pentobarbital (Nembutal) was purchased from Abbott Laboratories (Abbott Park, IL, USA). All other chemicals were of analytical grade.

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Cells

MDCKII (Madine-Darby Canine Kidney) parental cell line and MDCKII cells overexpressing ABCB1 (MDCKII-ABCB1), ABCG2 (MDCKII-ABCG2), ABCC2 (MDCKII-ABCC2) and ABCC5 (MDCKII-ABCC5) were provided by the Netherlands Cancer Institute (dr. A. Schinkel) and cultured in DMEM complete high glucose medium with L-glutamine supplemented with 10% FBS.

Animals

All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. Pregnant Wistar rats were purchased from Meditox Ltd. (Konarovice, Czech Republic) and were maintained in 12/12-h day/night standard conditions with water and pellets ad libitum. Experiments were performed on day 21 of gestation. Fasted rats were anesthetized with pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL) in a dose of 40 mg/kg administered into the tail vein.

Transport experiments *in vitro*

MDCKII-parental and MDCKII cells overexpressing ABCB1, ABCG2, ABCC2 or ABCC5 were seeded at a density of 1.5×10^6 cells per well and cultured for 3-4 days to confluence with daily medium replacement. Transport assays were performed on microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, NY) for two kinds of experimental conditions, as described below. The final activity

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of [^{3}H]-AZT was 0.04 μCi/ml and was tested at a low concentration of 5 nM as dictated by the specific activity of radioisotopes required for analysis.

Bidirectional transport assays were used to quantify AZT transport across cell monolayers in the apical-to-basolateral (AB) and basolateral-to-apical (BA) directions, as described in our previous studies [32, 33]. One hour before starting the experiment, cells were washed with PBS (37 ºC) and incubated with 2 mL of Opti-MEM (Invitrogen, Carslbad, CA) either alone or containing the dual ABCB1 and ABCG2 inhibitor elacridar (2 μM) [33], 50 μM AZT, or 500 μM lamivudine. The experiment was started by replacing the medium in the donor compartment (either apical or basolateral) with fresh Opti-MEM (37 ºC) containing [^{3}H]-AZT alone or with elacridar (2 μM), AZT (50 μM) or lamivudine (500 μM). AZT at 50 μM concentration was used in accordance with recommendations for *in vitro* evaluation of substrates of ABC transporters published by FDA in Guidance for Industry [34]. Lamivudine was used in order to investigate drug-drug interactions with AZT on the tested transporters at high concentration. Aliquots of 50 μl were collected at 2, 4 and 6 h from the acceptor compartment and radioactivity was measured by liquid scintillation counting (Tri-Carb 2900 TR Perkin Elmer). The percentage of radioactivity in the acceptor compartment relative to the stock solution initially added to the donor compartment was calculated. Ratios of BA to AB translocation after 6 h incubation (r_t) were also calculated [22].

To evaluate AZT active transport in more sensitive concentration equilibrium transport assays [35] we used the same cell cultures and seeding conditions as in the bidirectional transport assays. After 1 h preincubation period, the transport study was initiated by adding [^{3}H]-AZT in the presence or absence of elacridar (2 μM) or lamivudine (500 μM) to both (apical and basolateral) sides of the monolayer to give the same initial AZT concentration in both compartments. Aliquots of 50 μl were collected at 2, 4 and 6 h from both compartments

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and radioactivity was measured by liquid scintillation counting. The percentage of radioactivity in the apical and basolateral compartments relative to the initial stock solution was calculated [35]. Ratios of the percentages reached after 6 h incubation (r_e) were calculated.

In both *in vitro* setups, leakage of FITC-dextran was analyzed at the end of the experiments and deemed acceptable up to 1% per hour.

Dual perfusion of the rat placenta

The method of dually perfused rat term placenta was used as described previously [33, 36]. In brief, one uterine horn was excised and submerged in heated Ringer's saline. A catheter was inserted into the uterine artery proximal to the blood vessel supplying a selected placenta and connected with the peristaltic pump. Kreb's perfusion liquid containing 1% dextran was brought from the maternal reservoir at a rate 1 ml/min. The uterine vein, including anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighbouring fetuses by ligatures. The umbilical artery was catheterized by use of a 24-gauge catheter connected to the fetal reservoir and perfused at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner and the selected fetus was removed. Before the start of each experiment, the fetal vein effluent was collected into preweighed glass vials to check for a possible leakage of perfusion solution from the placenta. In the case of leakage, the experiment was terminated. Maternal and fetal perfusion pressures were maintained at levels close to physiological values and monitored continuously throughout the perfusion experiments as described previously. [^{3}H]-AZT concentration used in this experimental setup (9 nM) responds to the final activity of 0.06 μCi/ml that was necessary for radiochemical analysis.

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Two types of placenta perfusion systems were used in this study:

Open-circuit perfusion system was employed to study fetal-to-maternal (F→M) and maternal-to-fetal (M→F) AZT clearances. [^{3}H]-AZT (9 nM) was added to either maternal (M→F studies) or fetal (F→M studies) reservoir immediately after successful surgery. After 5-min stabilization period the sample collection started (time 0). Fetal effluent was sampled into preweighed vials in 5 min interval, concentration was measured radiometrically and transplacental clearance was calculated from all measured intervals as described below. At the end of experiment, placenta was perfused with radioactivity-free buffer for 10 min, excised from the uterine tissue and dissolved in tissue solubilizer (Solvable; PerkinElmer Life and Analytical Sciences) and its radioactivity was measured to detect tissue bound AZT (Tri-Carb 2900 TR Perkin Elmer).

Closed-circuit (recirculation) perfusion system was employed to further study involvement of placental transporter(s) in AZT transplacental transport. Both maternal and fetal sides of the placenta were infused with equal concentrations of [^{3}H]-AZT (9 nM) and after a short-time stabilization period, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (250 µl) were collected every 10 min from the maternal and fetal reservoirs and concentration of tested compound was measured. This experimental setup ensures steady concentration on the maternal side of the placenta and enables investigation of fetal/maternal ratio; any net transfer of the substrate implies transport against a concentrate gradient and provides an evidence of active transport. Effect of elacridar (2 µM) and indomethacin (0.28 mM) on AZT transport was investigated to identify transporters modulating AZT placental passage. AZT concentration of 5 µM corresponds with peak plasma concentration in pregnant women [37] and 500 µM concentration was used to detect saturability of the transport system. Lamivudine

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was added into perfusion solution in order to reveal possible drug-drug interactions on ABC transporters.

Pharmacokinetic analysis of efflux transport activity in the placenta

Organ clearance was applied to mathematically describe AZT M→F and F→M transport in open-circuit perfusion system. Averaged data from the intervals of 10 – 35 min were used for the following calculations. M→F transplacental clearance (Cl_{mf}) normalized to placenta weight was calculated according to Equation. 1.

$$Cl_{mf} = \frac{C_{fv}.Q_f}{C_{ma}.W_p}$$

where C_{fv} is concentration of tested compound in the umbilical vein effluent, Qf is the umbilical flow rate, Cma is concentration of AZT in the maternal reservoir and Wp is the wet weight of the placenta. F→M transplacental clearance (Cl_{fm}) was calculated according to Equation 2.

$$Cl_{fm} = \frac{(C_{fa} - C_{fv})Q_f}{C_{fa}.W_p}$$

where Cfa is concentration of tested compound in the fetal reservoir entering the perfused placenta via the umbilical artery.

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Results

AZT transport across cell monolayers: bidirectional transport assays

We first determined the transepithelial transport of [^{3}H]-AZT (5 nM) across monolayers of parental and ABCB1-, ABCG2-, ABCC2- and ABCC5-overexpressing cells. No asymmetry in BA vs. AB transport of AZT was observed in MDCKII-parental cells and cells transduced with ABCC2 and ABCC5 as obtained r_t were in the range of 0.9 - 1.1 (Fig 1A, 1B, 1C). In contrast, significantly larger r_t were observed in ABCB1 and ABCG2 overexpressing cells (r = 2.2 and 2.4, respectively, Fig 1D, 1H). Addition of a dual ABCB1/ABCG2 inhibitor elacridar (2 μM) completely abolished this asymmetry, yielding r_t of approximately 1 (Fig 1E, 1I). These findings suggest that AZT is a substrate of human ABCB1, ABCG2 but not ABCC2 or ABCC5 transporters. At higher AZT concentrations (50 μM), we observed reduced transport ratio ($r_t < 2$) (Fig 1F, 1J) suggesting saturation of the transport system. Lamivudine (500 μM) affected neither ABCB1- nor ABCG2-mediated AZT transport, thus indicating zero interactions (Fig 1G, 1K).

AZT transport across cell monolayers: concentration equilibrium transport assays

As AZT is a lipophilic compound, we used highly sensitive concentration equilibrium transport assays [35] to further confirm its transport across the cell monolayer. Under concentration equilibrium conditions, increased AZT concentrations in the apical compartment and decreased AZT concentrations in the basolateral compartment were observed after 6 h incubation in MDCKII-ABCB1 and MDCKII-ABCG2 cells (Fig 2A, 2D). Addition of ABCB1/ABCG2 inhibitor elacridar (2 μM) completely blocked active AZT transport ($r_e \approx 1$), confirming that ABCB1 and ABCG2 affect AZT transport across the cell

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membrane (Fig 2B, 2E). Lamivudine (500 μM) did not influence AZT transport as (r_e) was not significantly altered (Fig 2C, 2F). These findings suggest no drug-drug interactions between AZT and lamivudine on ABCB1 or ABCG2 transporters. In MDCKII cells overexpressing ABCC2 and ABCC5, no change in AZT concentration was observed in either the apical or basolateral compartments ($r_e \approx 1$), confirming no active transport in these cells (Fig 2G, 2H).

Open circuit perfusion experiments: transplacental clearances of AZT in M→F and F→M directions

Maternal or fetal side of the placenta were infused with [^{3}H]-AZT at 9 nM concentration and total transplacental clearance was subsequently detected. The mean F→M clearance was higher than that in the opposite direction, however without statistical significance (Fig 3) suggesting minor role of active mechanism in AZT transplacental transport. Less than 1 % of AZT dose was detected in the placenta after perfusion experiments, indicating limited tissue binding and negligible effect on clearance calculation.

Closed circuit perfusion experiments: effect of concentrations and inhibitors on AZT transport across the placenta

To compare fetal and maternal AZT concentrations at steady-state, both sides of the placenta were perfused with AZT in closed circuit experimental setup. At 9 nM and 5 μM concentrations significant decrease in AZT concentration in the fetal perfusate was observed indicating active transport against concentration gradient from fetal to maternal side of the placenta. The decline was blocked by elacridar (2 μM) and indomethacin (0.28 mM) and

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saturated at high AZT concentrations (500 μM). In contrast, co-administration of lamivudine (500 μM) had no effect on AZT transport thus excluding interactions between these two drugs on ABC placental transporters (Fig 4).

Discussion

The use of AZT-containing antiretroviral therapy represents an important advance in preventing mother-to-child transmission of HIV infection. The risk, however, encompasses numerous long-term toxicities such as hematological and cardiac complications that have been observed in children exposed to AZT *in utero* [4-7]. To be able to minimize the risk of toxicity affecting newborns it is of particular interest to understand all factors potentially affecting AZT disposition between mother and fetus. Since AZT is commonly given in combination with other antiretroviral drugs, it is also important to consider possible drug-drug interactions on membrane transporters that may modulate placental transfer of drugs and increase fetal exposure to xenobiotics [9]. Physical-chemical properties of AZT (logP 0.10) indicate its rapid membrane passage by the mechanism of passive diffusion [38]. On the other hand AZT disposition into target compartments was found to be controlled by efflux as well as influx membrane transporters [21, 22, 27, 39, 40]. In the present study we aimed to identify drug efflux ABC transporters affecting AZT placental transport and to evaluate possible drug-drug interactions between AZT and lamivudine, another antiretroviral drug frequently co-administered with AZT.

To screen whether AZT is a substrate of selected human ABC transporters we used the well-established MDCKII cell line model recommended by the International Transporter Consortium [41]. It is accepted that potential substrates of ABC transporters reach the transport BA/AB ratio of at least 2 and this transport can be inhibited by model inhibitors by

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50% [41]. Based on our results we suggest AZT as a substrate of human ABCB1 and ABCG2 (r_t were 2.2 for ABCB1 and 2.4 for ABCG2, Fig 1D, 1H) and we provide the first evidence that AZT is not a substrate of ABCC2 or ABCC5 (Fig 1B, 1C). MDCK/MDCKII-ABCB1 cell line model has been previously used to detect AZT interactions with human ABCB1 with inconclusive results [24, 25]. Shaik et al. observed that ABCB1-overexpression in MDCKII cells had no effect on AZT transport or accumulation and concluded lack of ABCB1-mediated efflux of this compound [25]. On the contrary, de Souza et al. detected significant asymmetry in transport of AZT across the MDCK-ABCB1 overexpressing cells that was blocked by elacridar proposing AZT be a substrate of ABCB1 [24]. The lack of AZT interaction with ABCB1 observed by Shaik et al. [25] might be explained by the experimental setup employed as the authors evaluated bidirectional AZT transport after 90 min which might be too a short period for decisive conclusions. In our experiments we were not able to detect active AZT transport after two hours; however, 6 hour transport experiment revealed significant asymmetry in AZT transport across ABCB1-overexpressing cells. Our data thus confirm those of de Souza et al. [24]. Considering AZT interactions with human ABCG2 transporter, our findings are in line with observations by Wang et al. who reported lower accumulation of AZT in CD4+ T-cell line overexpressing ABCG2 sensitive to specific ABCG2 inhibitor fumitremorgin C [28]. Application of higher doses of AZT (50 μM) reduced the $r_t < 2$ suggesting partial saturation of both ABCB1 and ABCG2 transport capacity (Fig 1F, 1J). In our *in vitro* experimental setup ABCC2 and ABCC5 did not recognize AZT as a substrate. The lack of AZT interaction with ABCC5 is contradictory to data observed by Jorajuria et al. [26], where ABCCs inhibitor probenecid significantly increased AZT anti-HIV activity in human macrophages. However, involvement of other transporters mediating AZT transport, such as organic anion transporters that are known to be inhibited by probenecid as well [34] cannot be excluded.

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To strengthen the outcomes of the *in vitro* bidirectional transport study we employed the concentration equilibrium assays on cell monolayers, which is a more suitable method when investigating interaction of highly hydrophobic compounds with ABC transporters [35]. Using this experimental setup we further confirmed that AZT is actively transported across the cell monolayer by ABCB1 and ABCG2 but not by ABCC2 or ABCC5 (Fig 2A, 2D, 2G, 2H). In both *in vitro* experimental setups the presence of elacridar, a dual ABCB1/ABCG2 inhibitor, completely blocked active AZT transport confirming specificity of the transport observed (Fig 1E, 1I and Fig 2E, 2H). In contrast, lamivudine had no effect on AZT passage across the cell monolayer suggesting that these drugs do not compete for ABCB1 and/or ABCG2 mediated transport (Fig 1G, 1K and Fig 2C, 2F).

To further investigate interactions of AZT with ABC transporters on the organ level, we employed the method of dually perfused rat term placenta, which is a well justified and frequently used model for assessing transplacental pharmacokinetics because of similar ABC drug efflux transporter expression, localization and function in human and rodent placentas [9, 10, 13, 36, 42]. Comparing M→F and F→M clearances in open-perfusion setup we observed only insignificant asymmetry in AZT transport at non-saturating concentration suggesting minor role of ABC transporters in AZT transport across the placenta (Fig 3). This is in accordance with AZT transport in human perfused placenta where no active transport was detected [18, 20, 43]. It can be speculated that the limited effect of ABC transporters on AZT placental transport is due to high lipid solubility of the compound that overshadows the activity of the transporter(s) [9]. Nevertheless, under concentration equilibrium experimental setup in which both sides of the placenta were infused with the same AZT concentrations, we detected significant decrease in AZT concentration on the fetal side suggesting AZT can cross the placenta against concentration gradient in F→M direction. This active transport was inhibited by elacridar indicating involvement of ABCB1/ABCG2 (Fig 4). When increasing

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inflow AZT concentrations to 5 μM, which corresponds with therapeutic levels [37], no effect on transport activity was observed. However, at 500 μM AZT concentration complete saturation of the active transport was detected (Fig 4). When indomethacin, a non-specific ABCCs inhibitor [44], was used (Fig 4) in perfusion studies, we observed significantly reduced active AZT transport across the rat placenta. Considering our *in vitro* results and Sai et al. study [39] in which rat Abcc(s) did not participate in AZT placental transport, we propose indomethacin effect on AZT transport could be mediated by either inhibition of organic anion transporter 4 [45] that recognizes AZT as a substrate [46] or by other so far unidentified mechanism.

Drug-drug interactions between AZT and acyclovir on placental transporters were suggested by Brown et al. [47] who compared pharmacokinetics and placental transfer of these drugs in pregnant rats; when administered in combination acyclovir showed a significant threefold increase in drug exposure in amniotic fluid and fetal tissues whereas AZT exposure tended to decrease. The authors proposed involvement of transporter system responsible for the altered pharmacokinetics of both drugs [47] and as acyclovir has been suggested to be an ABCB1 substrate [48] it can be speculated that this interaction might be at least partially mediated by ABCB1. Interestingly, although lamivudine is supposed to interact with ABCB1/ABCG2 [23, 24, 30], we did not observe any effect of lamivudine on AZT transport *in vitro* or *in situ*. However, our so far unpublished data question ABCB1/ABCG2-mediated transport of lamivudine and we, therefore, propose that AZT and lamivudine do not compete for these transporters in their placental transfer.

In our experimental setups, we used radiolabelled AZT for fast detection. Since neither MDCK cells [22], nor placenta [43] possess high capacity to metabolise the compound, we are confident that we detected the parent compound and no metabolites contaminated our

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data and calculations. Our findings suggest that placental ABCB1/ABCG2 transporters have weak (if any) influence on AZT disposition between mother and fetus at term. However, placental transport function undergoes substantial changes during placental development and altered expression and function of transporters can be one of the key factors modulating fetal drug exposure [49]. Our findings are of importance in situations in which AZT is combined with other substrates/inhibitors of ABC transporters which may lead to larger fetal exposure and higher risk of detrimental effects on fetus {Daud, 2015 #111}. Nevertheless, further evidence is required in order to establish personalized combination therapies for pregnant women.

Conclusion In summary we propose that AZT is a substrate of human ABCB1 and ABCG2 transporters but not of ABCC2 or ABCC5. We observed the effect of human ABC transporters on AZT transcellular passage; involvement of these transporters in AZT transplacental pharmacokinetics in rats was observed under concentration equilibrium conditions only. Importantly, we propose that frequently used combination of AZT and lamivudine does not possess a risk of drug-drug interactions on ABC transporters. However, possible interactions should be presumed when adding another antiretroviral drug into combination with AZT as it could potentially increase placental transport and fetal toxicity of co-administered substrates of these transporters.

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Acknowledgements

Z.N. performed the *in situ* placenta perfusion experiments and analyzed the data, participated in the *in vitro* cell-based experiments and wrote the manuscript; L.C. designed and performed the *in vitro* cell-based experiments, analyzed the *in vitro* data and participated in writing the manuscript. M.C. participated the *in vitro* cell-based experiments and assisted with writing the manuscript F.S. supervised the experiments, critically revised the manuscript and secured funding for the project. This research was financially supported by the Czech Science Foundation (GACR P303/120850 and P303/13-31118P) and by Charles University in Prague (grant No. SVV/2015/260-185). We wish to thank Dana Souckova and Renata Exnarova for skillful assistance with the perfusion experiments.

Conflict of Interest

The authors have no conflict of interest to declare.

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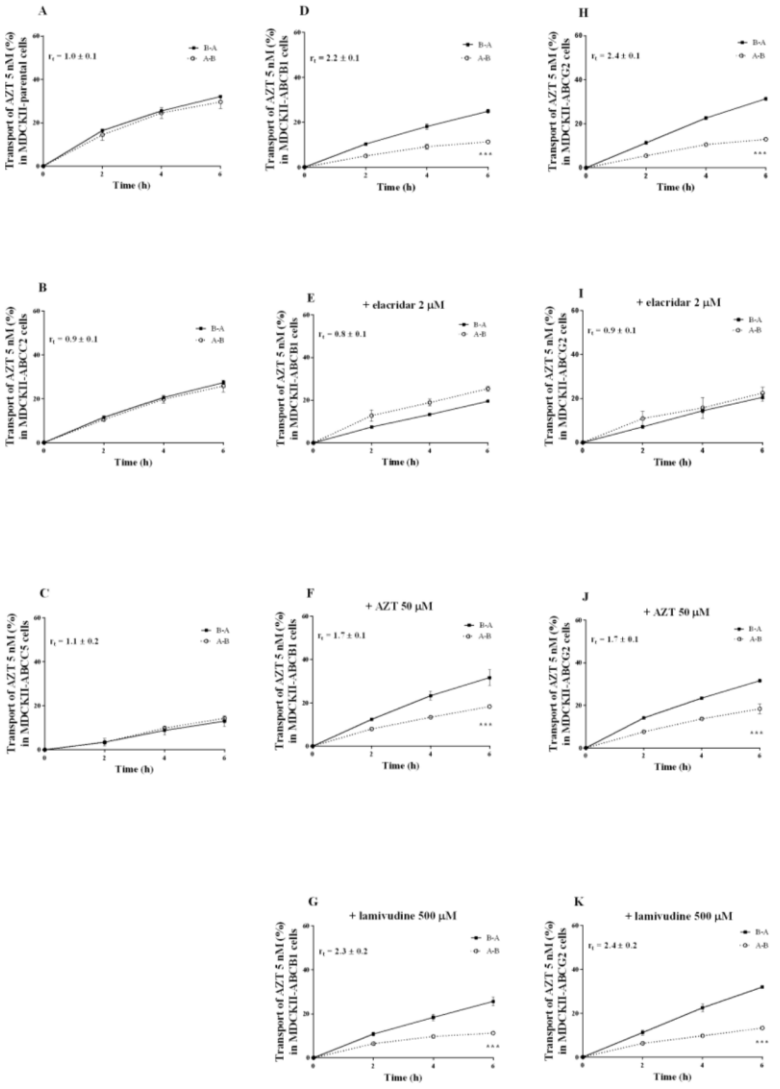

Figure 1 AZT transport in MDCKII-parental (A), ABCC2- (B), ABCC5-(C), ABCB1-(D, E, F, G) and ABCG2-overexpressing (H, I, J, K) cells after 6h incubation using bidirectional transport assay.

AZT transport in MDCKII parental and ABCC2-, ABCC5-, ABCB1, and ABCG2-overexpressing cells after 6 h incubation was detected using bidirectional transport assay. AZT at 5 nM concentration was added to the donor compartment (apical or basolateral) and samples of 50 µl were collected at 2, 4 and 6 h from the acceptor compartment. The percentage of radioactivity in the acceptor compartment relative to the stock solution initially added to the donor compartment was calculated and the transport ratio (r_t) of BA to AB translocation after six hours was determined. AZT bidirectional transport assay was assessed in the absence (A, B, C) or presence of elacridar 2 µM (E, I), unlabeled AZT 50 µM (F, J) or lamivudine 500 µM (G, K) in both compartments. Asymmetry in AZT transport indicating active transport across the monolayer was observed in ABCB1- and ABCG2-overexpressing cells (r_t = 2.2 and 2.4, respectively) but not in parental (r_t = 1.0), ABCC2- (r_t = 0.9) and ABCC5-overexpressing cells (r_t = 1.1). Elacridar completely abolished AZT active transport. AZT (50 µM) reduced transport ratios (r_t < 2) and lamivudine had no effect on ABCB1/ABCG2 mediated AZT transport. Data points are means (n = 3) ± SD, Student's t-test was employed to compare AZT transported amount at six hour time point (***$p < 0.001$).

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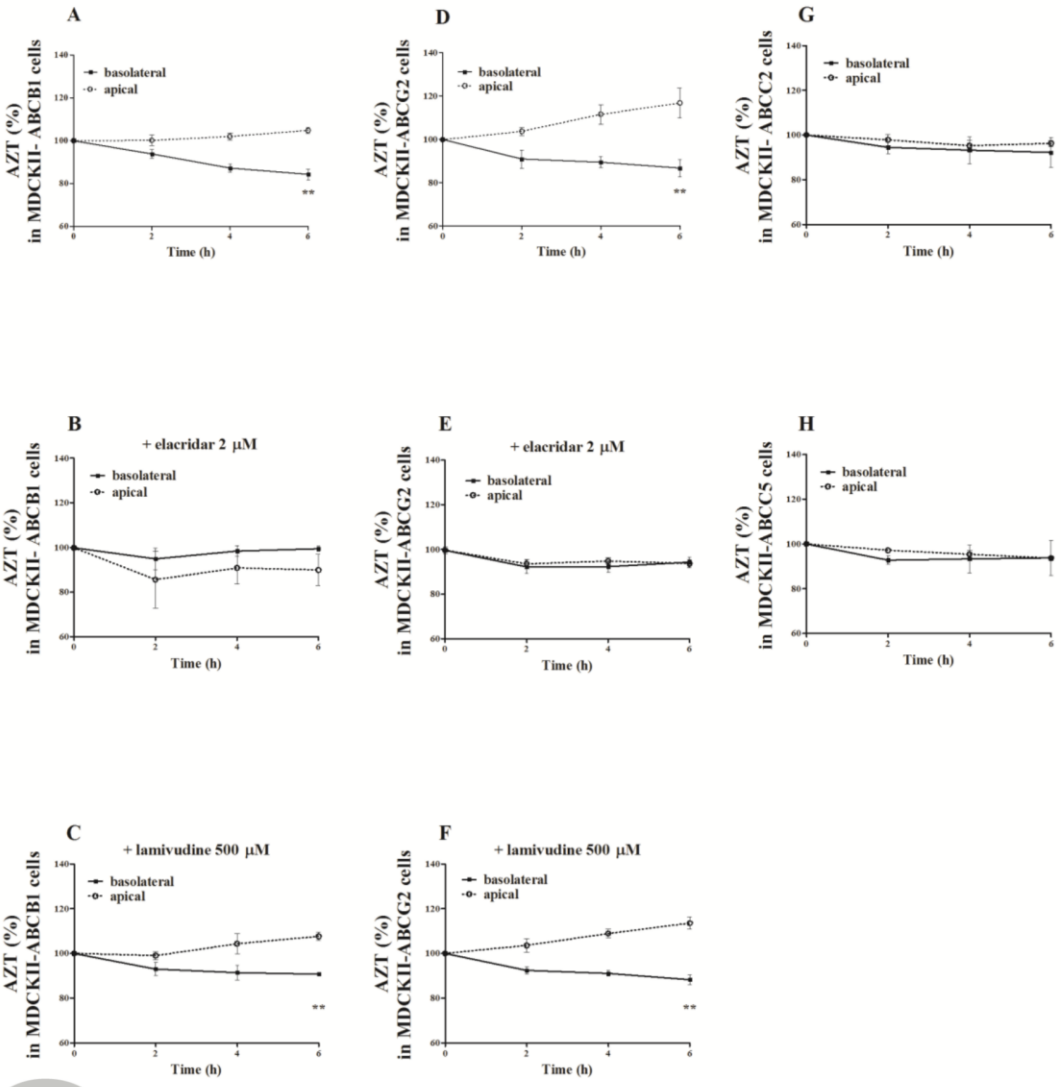

Figure 2 AZT transport in MDCKII-ABCB1 (A), ABCG2-(D), ABCC2-(G) and ABCC5-overexpressing (H) cells after 6h incubation in the absence of inhibitor or in the presence of elacridar (B, E) or lamivudine (C, F) under concentration equilibrium conditions.

AZT at 5 nM concentration was added to the basolateral and apical compartments and samples were obtained from both compartments (A, D, G, H). ABCB1/ABCG2 inhibitor elacridar at 2 µM concentration (B, E) or lamivudine at 500 µM concentration (C, F) were added to the both compartments at the beginning of experiment. Data are presented as the percentage of AZT concentration of the initial AZT concentration (= 100 %) in the apical or basolateral compartments. Ratios of the percentage reached after 6 h incubation were calculated. In MDCKII-ABCB1 and ABCG2-overexpressing cells, increased AZT concentrations in the apical compartments and decreased AZT concentrations in the basolateral compartments were observed after 6 h incubation indicating active AZT transport mediated by ABCB1 and ABCG2. This active transport was completely abolished in the presence of elacridar but not lamivudine. In MDCKII-ABCC2 and ABCC5-overexpressing cells no change in AZT concentration was observed confirming no ABCC2/ABCC5-mediated AZT transport. Data points are means (n = 3) ± SD, Student's t-test was employed to compare AZT concentrations in the apical and basolateral compartments measured at six hour time point (**$p < 0.05$).

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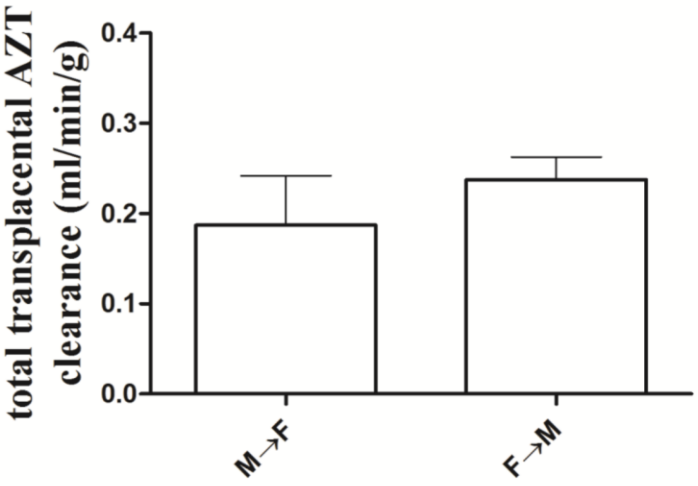

Figure 3 AZT transport across dually perfused rat term placenta in the open experimental setup. AZT at 9 nM concentration was added to either maternal or fetal compartment and AZT concentration was measured in fetal venous outflow. Total transplacental M→F and F→M clearances were calculated by Eq. 1 and 2 (see Materials and methods), respectively. No significant difference was detected between M→F and F→M clearances. Data are presented as means ± SD n ≥ 3. Student's t-test was used in order to evaluate statistical significance of differences between clearances.

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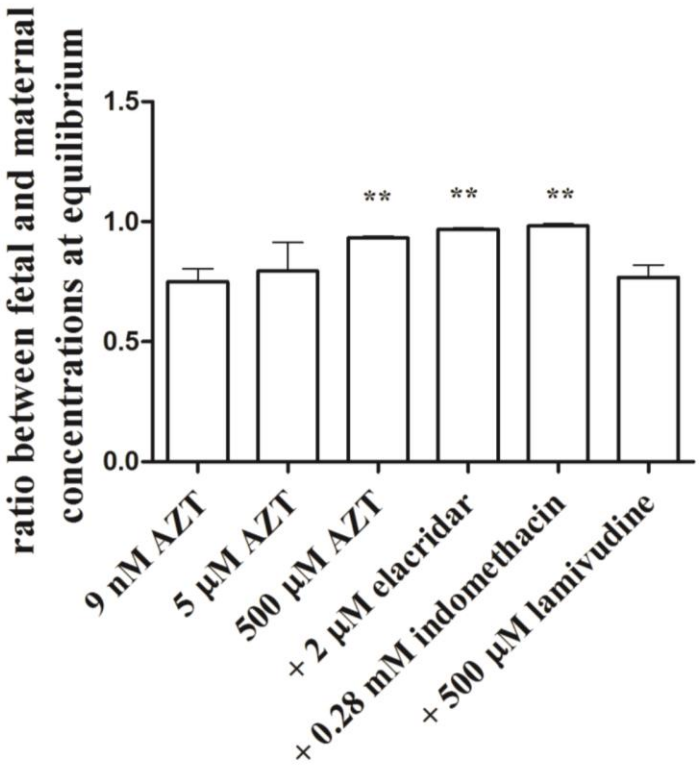

Figure 4 AZT transport across dually perfused rat term placenta in the closed experimental setup. Both fetal and maternal sides of the placenta were simultaneously infused with 9 nM AZT. Fetal perfusate was recirculated for 60 min and at the end of experiment, fetal and maternal concentrations of AZT were compared. Decrease in AZT concentration in the fetal perfusate was observed. This decrease was significantly inhibited by elacridar (2 µM) and indomethacin (0.28 mM) and saturated when 500 µM concentration of cold AZT was added into perfusate. Conversely lamivudine (500 µM) and cold AZT (5µM) had no effect. Data are presented as means ± SD n ≥ 3, statistically significant different from 9 nM AZT (ANOVA followed by Bonferroni's test, $**p < 0.05$).

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7.4 Long-term administration of tenofovir or emtricitabine to pregnant rats; effect on *Abcb1a, Abcb1b,* and *Abcg2* expression in the placenta and in maternal and fetal organs

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Léčiva TFV a emtricitabin zvolená v naší studii jsou vysoce účinná antiretrovirotika s příznivým bezpečnostním profilem, která jsou v současnosti jedny z nejpoužívanějších v rámci terapie HIV infekce u těhotných žen. Cílem této práce bylo zjistit, zda dlouhodobé podávání TFV nebo emtricitabinu má vliv na expresi dvou hlavních ABC transportérů – *Abcb1a, Abcb1b* a *Abcg2* to v různých tkáních matky i plodu.

Potkanům kmene Wistar byl od 12. do 21. gestačního dne i.m. aplikován roztok TFV (2.25 mg/kg/den), emtricitabinu (3.5 mg/kg/den) nebo chloridu sodného (0,9 %). Na konci březosti (22. den gestace) byla zvířata usmrcena a matkám i plodům byly odebrány vybrané orgány, u nichž byla následně provedena expresní analýza. Plody a jejich placenty byly zváženy a byl stanoven poměr hmotnosti placenty vůči hmotnosti plodu jakožto indikátor míry rizika onemocnění kardiovaskulárními chorobami v dospělém věku.

Bylo zjištěno, že dlouhodobá aplikace obou antiretrovirotik nevede k signifikantním změnám exprese žádného z testovaných transportérů ve střevě, mozku, ledvinách a játrech matky či plodů a ani v placentě. Zajímavé je, že aplikace obou látek vedla ke zvýšení poměru váhy placenty/plodu, v případě TFV byla tato změna signifikantní. Naše data přispívají k celkovému zhodnocení bezpečnostního profilu obou látek.

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Research Paper

Long-term administration of tenofovir or emtricitabine to pregnant rats; effect on *Abcb1a*, *Abcb1b* and *Abcg2* expression in the placenta and in maternal and fetal organs

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Keywords
breast cancer resistance protein; drug interactions; emtricitabine; P-glycoprotein; pregnancy; tenofovir

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Abstract

Objectives Tenofovir and emtricitabine are very effective and well-tolerated antiretrovirals representing current backbone of the antiretroviral combination regimens for the prevention of perinatal HIV transmission. The aim of our study was to determine whether tenofovir or emtricitabine administered in long-term fashion affect expression of two widely described pharmacokinetic determinants, P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2), in maternal or fetal biological tissues.

Methods For this purpose, pregnant Wistar rats were administered tenofovir (2.25 mg/kg/day), emtricitabine (3.5 mg/kg/day) or saline i.m. for 10 days (from the 12th to 21st gestation day). On the 22nd day, the placenta and maternal/fetal intestine, brain, kidneys and liver were sampled and analysed for *Abcb1a*, *Abcb1b* and *Abcg2* expression; placental and newborns' weights were also monitored.

Key findings We found that long-term application of tenofovir or emtricitabine did not significantly affect expression of *Abcb1a*, *Abcb1b* and *Abcg2* in either maternal or fetal organs. However, tenofovir administration significantly increased placenta-to-birthweight ratio, a strong indicator of various diseases occurring later in life.

Conclusions Our data broaden current knowledge on safety profile of tenofovir and emtricitabine use in pregnancy. Nevertheless, further research in other mammal species, including humans, is important to fully elucidate this issue.

Introduction

Combination antiretroviral therapy (cART) is used in pregnant women to treat HIV infection and to prevent perinatal HIV transmission. cART is preferred to monotherapy as it is more effective, decreasing the rate of perinatal transmission from approximately 20–30% to 0.1–0.5%[1,2] while preserving lower risk of developing drug resistance.[3]

Searching for highly effective and safe cART utilizable in pregnancy is a constantly evolving process that reflects the progress in clinical and basic research.[4] Currently, nucleotide/nucleoside reverse transcriptase inhibitors, tenofovir (orally administered in the form of disoproxil fumarate) and emtricitabine, belong to the backbone of antiretroviral regimens for prevention of perinatal HIV transmission recommended by the US National Institute of Health.[3] Both drugs are combined with other antiretrovirals based on the individual treatment requirements.[3] Despite growing body of evidence on good tolerability and efficacy of tenofovir and emtricitabine use in pregnancy,[5,6] animal studies indicate the possibility of fetal low birthweight resulting in a tendency to hypertension or decreased bone density.[7,8]

Pharmacokinetics of many antiretrovirals is affected by the activity of two important drug efflux pumps – P-glycoprotein (ABCB1) or breast cancer resistance protein (ABCG2).[9–11] Both transporters are highly expressed in the intestine, liver and kidneys, thus affecting the absorption and excretion of their substrates.[12,13] ABCB1 and ABCG2 also protect sensitive tissues such as brain and developing fetus as they limit penetration of xenobiotics across the blood–brain and placental barrier, respectively.[12,13] Most of studies on ABCB1 and ABCG2 have

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been performed in adults so far; nevertheless, those carried out in fetuses or neonates have confirmed that functional ABCB1 and ABCG2 in the fetal blood–brain barrier are exceptionally important for protection of the developing brain.[14,15]

To guarantee safe and efficient therapy, drug–drug interactions on ABCB1 and ABCG2 transporters have to be always borne in mind when treating comorbidity in HIV-positive patients or when considering new cART regimen.[9,13] To date, many studies have shown that tenofovir and emtricitabine are not substrates/inhibitors of ABCB1 or ABCG2.[10,16–18] Conversely, data describing capability of tenofovir or emtricitabine to alter ABCB1 or ABCG2 expression are rather sparse and originate from in-vitro short-term testing.[19–21] More importantly as both drugs cross the placenta into fetal circulation,[10,22] their effects on expression of ABCB1 and ABCG2 in fetal tissues also remain to be elucidated.

Therefore, the aim of our study was to investigate whether tenofovir or emtricitabine administered in a long-term fashion during pregnancy may modulate the expression of *Abcb1a*, *Abcb1b* and *Abcg2*, the rat orthologues of the human genes, in the maternal and fetal organs (brain, intestine, kidneys and liver) and in the placenta. For this purpose, we treated pregnant Wistar rats with tenofovir, emtricitabine or saline for 10 days (from the 12th to 21st gestation day). On the 22nd gestation day, the tested organs were collected for relative expression analysis of *Abcb1a*, *Abcb1b* and *Abcg2*. Placenta and newborns' weights were also monitored at term and placenta-to-birthweight ratio was calculated as a predictor of various diseases occurring later in life.[23–27]

Methods

Reagents and chemicals

Tenofovir and emtricitabine were kindly provided by Gilead Sciences, Inc., Foster City, CA, USA. Radiolabelled [adenine-2,8-^{3}H]-tenofovir ([^{3}H]tenofovir) and [6-^{3}H]-emtricitabine ([^{3}H]emtricitabine) were purchased from 4 Moravek Biochemicals, CA, USA. Pentobarbital (Nembutal) was purchased from Abbott Laboratories (Abbott Park, IL, USA). All other chemicals were of analytical grade.

Animals

Female Wistar rats were used for all the experiments. Non-pregnant and pregnant rats were purchased from MediTox (Konarovice, Czech Republic) and maintained at 12/12-h day/night standard conditions with water and pellets *ad libitum*. All experiments were approved by the ethical committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were carried out in accordance with the Guide for The Care and Use of Laboratory Animals (1996) and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986). Gestation day was counted since detection of a copulatory plug of sperm after overnight mating.

Bioavailability studies

To be able to estimate the exposure of animals to tenofovir or emtricitabine, we first characterized their bioavailability after i.m. administration. Bioavailability experiments were carried out in separate non-pregnant animals. Fasted rats ($n = 2$) were anesthetized with pentobarbital at the dose of 40 mg/kg administered into the tail vein. Single dose of tenofovir (2.25 mg/kg) or emtricitabine (3.5 mg/kg) traced with [^{3}H]tenofovir or [^{3}H]emtricitabine (15 μCi/ml), respectively, at the final volume of 0.2 ml was applied into back side of the tight muscle for i.m. and into femoral vein for i.v. administration. Blood samples (0.3 ml) were collected into heparinized tubes from the jugular vein 2, 5, 15, 30, 60, 120 and 240 min after application. Blood samples were decolourized using SOLVABLE^{TM} (PerkinElmer, Waltham, MA, USA) according to the manufacture instructions. Radioactivity was analysed employing Tri-Carb 2900 TR PerkinElmer (PerkinElmer). Area under the curve between 0 and 240 min ($AUC_{0–240}$) was calculated by the trapezoidal method using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

Administration of tenofovir and emtricitabine to pregnant Wistar rat

Rats with positive copulatory plug of sperm after overnight mating ($n = 30$) were randomized in three groups ($n = 10$): tenofovir (2.25 mg/kg)-treated animals (tenofovir group), emtricitabine (3.5 mg/kg)-treated animals (emtricitabine group) and saline-treated group (control group). Saline or a tested drug (0.2 ml) was applied into the back side of the tight muscle from the 12th to 21st gestation day once daily. On the 22nd gestation day, animals not bearing fetuses were excluded from subsequent analysis and only pregnant animals (five in the control and emtricitabine group and four in the tenofovir group) were anesthetized with pentobarbital in a dose of 40 mg/kg administered into the tail vein. The average weights of pregnant rats on the 22nd day of gestation were 515.0 ± 32.8 g in the tenofovir group ($n = 4$), 412.0 ± 41.5 g in the emtricitabine group ($n = 5$) and 424.0 ± 65.3 in the control group ($n = 5$). Dams, their fetuses and their corresponding placentas were weighted and proceeded as follows: (1) the maternal kidneys, liver,

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brain and ileum and (2) two randomly selected placentas from each dam, (3) the kidneys, liver, brain and complete intestine were sampled from two randomly selected fetuses (pooled to one sample) of each dam. The organs were frozen in liquid nitrogen immediately after dissection and stored at −70 °C until analysis.

RNA isolation and real-time RT-PCR analysis

Expression of the rat *Abcb1a*, *Abcb1b* and *Abcg2* mRNA was analysed using quantitative RT-PCR (qRT-PCR) on iCycler Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Total RNA was isolated from weighed tissues using Tri Reagent solution (Molecular Research Centre) according to the manufacturer's instructions. The purity of the isolated RNA was verified by A_{260}/A_{280}, and RNA integrity was confirmed by electrophoresis on a 1% agarose gel. The concentration of RNA was calculated by A_{260} measurement. RNA (1 μg) was converted into cDNA using the Reverse Transcription master Mix (Generi Biotech Ltd, Hradec Kralove, Czech Republic). The reaction mixture contained 25 ng of the analysed cDNA. The amplification of each sample was performed in triplicate using gb Ideal PCR Master Mix (Generi Biotech Ltd.) and predesigned gb TaqMan PCR Gene Expression Assays for *Abcb1a* (rAbcb1a_Q1), *Abcb1b* (rAbcb1b_Q1) and *Abcg2* (rAbcg2_Q1). For greater precision, the data obtained were normalized to expression of two housekeeping genes analysed in each sample, *B2m* (beta-2-microglobulin) and *Ywhaz* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein).[28,29] Only when comparing expression of target genes between maternal and fetal organs, data were normalized to total RNA due to different expression of reference genes.[30,31] All PCR expression assay kits were purchased from Generi Biotech Ltd. The amplification of each sample was performed in triplicate. The time–temperature profile used was 95 °C for 3 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 25 s. Expression values of each sample were obtained using $2^{-\Delta\Delta ct}$ method as described previously.[32] Sensitivity of the expression arrays obtained was tested using ten time dilution series in range of 10^1–10^4 of cDNA prepared from placental RNA (data not shown).

Monitoring of placental and birthweight, analysis of placenta-to-birthweight ratio

On the 22nd gestation day, two randomly selected fetuses with their placentas from each animal in all tested groups were collected[33] and their weights averaged; that is, means derived from two fetuses or placentas were taken into downstream analysis reaching final $n = 5$ in the control and emtricitabine group and $n = 4$ in the tenofovir group.

Statistical analyses

Statistical significance was examined by unpaired nonparametric Mann–Whitney *U*-test or nonparametric Kruskal–Wallis followed by Dunn's *post hoc* test (GraphPad 6.0 Software, Inc.).

Results

Analysis of tenofovir and emtricitabine bioavailability after single dose i.m. administration

To estimate exposure of animals after i.m. administration, we first carried out bioavailability studies with both tenofovir (Figure 1a) and emtricitabine (Figure 1b). Tenofovir (2.25 mg/kg) and emtricitabine (3.5 mg/kg) showed bi-exponential decline after i.v. administration. AUC_{0-240} of both drugs after i.m. application were not different to those observed after i.v. administration reaching bioavailability (F) of i.m. administered tenofovir and emtricitabine ≈ 1 (Figure 1).

Semi-quantitative comparative analysis of *Abcb1a*, *Abcb1b* and *Abcg2* expression between maternal and fetal organs (brain, liver, kidneys and intestine) in the control group of animals

Abcb1a, *Abcb1b* and *Abcg2* were detectable in both fetal and maternal organs. *Abcb1a* showed significantly higher expression in the maternal brain, liver and kidneys when compared with its expression in respective fetal organs (Figure 2a); expression of *Abcb1a* was completely undetectable in one sample of fetal kidneys. Ontogenic expression of *Abcb1b* was also observed in the maternal liver and kidneys (Figure 2b); *Abcb1b* expression was completely undetectable in two samples originated from the fetal kidneys and liver. The levels of fetal and maternal *Abcg2* expression were comparable in all organs tested (Figure 2c). Due to different expression of reference genes, *B2m* and *Ywhaz*, between maternal and fetal organs expression of target genes was normalized to total RNA.

Effect of long-term administration of tenofovir and emtricitabine on *Abcb1a*, *Abcb1b* and *Abcg2* expression in the fetal organs (brain, kidneys, liver and intestine)

When analysing the effect of long-term exposure of pregnant rats to tenofovir (2.25 mg/kg/day) or emtricitabine (3.5 mg/kg/day) on *Abcb1a*, *Abcb1b* and *Abcg2* expression in the fetal organs, we observed trends of up- or

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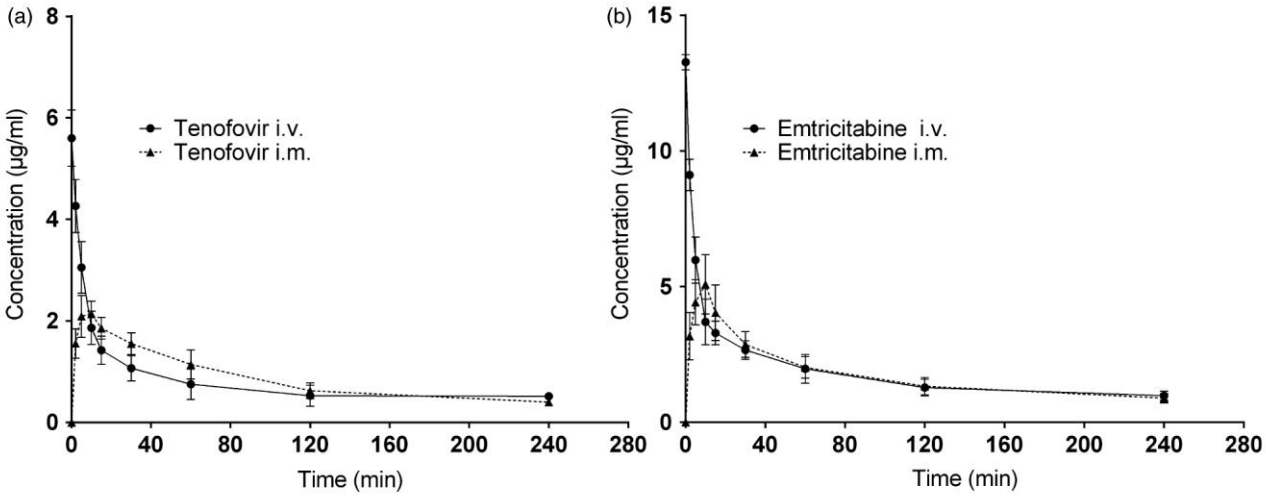

	Tenofovir		Emtricitabine	
Route of administration	i.v.	i.m	i.v.	i.m.
$AUC_{0\text{-}240}$ (µg×min/ml)	188.5 (±11.6)	208.4 (±16.7)	435.4 (±19.4)	420.1 (±15.4)
Bioavailability (F)		1.11 (±0.09)		0.96 (±0.04)

Figure 1 Pharmacokinetic profiles of (a) tenofovir (2.25 mg/kg) and (b) emtricitabine (3.5 mg/kg) after i.v. (●) and i.m. (▲) single-dose administration in non-pregnant Wistar rats. Each point represents the mean ± SD (n = 2). Area under the curve (AUC) after both types of administration was calculated in the range of 0 – 240 min.

down-regulation in certain cases but without statistical significance (Figure 3). The data obtained were normalized to two reference genes (*B2m* and *Ywhaz*), whose constitutive expressions in all the organs tested were confirmed (data not shown).

Effect of long-term administration of tenofovir and emtricitabine on *Abcb1a*, *Abcb1b* and *Abcg2* expression in the maternal organs (brain, kidneys, liver and proximal part of ileum) and placenta

Long-term exposure of pregnant rats to tenofovir (2.25 mg/kg/day) or emtricitabine (3.5 mg/kg/day) did not result in significant changes in expression of *Abcb1a* (Figure 4a), *Abcb1b* (Figure 4b) and *Abcg2* (Figure 4a) in the maternal organs or placenta. The data obtained were normalized to two reference genes (*B2m* and *Ywhaz*). Constitutive expression of *B2m* and *Ywhaz* in all organs tested was confirmed (data not shown).

Monitoring of placental weight, birthweight and analysis of placenta-to-birthweight ratio

In the tenofovir group, we observed significant increase in placental weight while birthweight was comparable in all tested animals; subsequently, placenta-to-birthweight ratio was significantly elevated (1.7 fold) when compared with control group (Figure 5). No effect on placenta or birthweight was observed in the emtricitabine group.

Discussion

The latest clinical trials focused on tenofovir and emtricitabine use in HIV-positive pregnant women have shown their favourable risk-benefit profile[5,6,34,35] and, therefore, both have been increasingly used in cART during the past decade.[6,36] However, experiments in pregnant animals indicate potential risk of their use during pregnancy.[7,8]

Many studies using various experimental approaches have shown lack of interactions between tenofovir or emtricitabine and ABCB1 or ABCG2 transporters.[10,16–18] On the other hand, data on tenofovir or emtricitabine effects on ABCB1/ABCG2 expression are rather sparse and were generated using in-vitro models or short-term exposure.[19–21] Here, we investigated the effect of long-term administration of tenofovir or emtricitabine during gestation on *Abcb1a*, *Abcb1b* and *Abcg2* expression in maternal and fetal tissues *in vivo*.

It is impossible to simulate transplacental transfer and effects of antepartum therapy on expression of maternal/fetal genes *in vitro*; neither can this information be adequately, ethically and safely acquired through other

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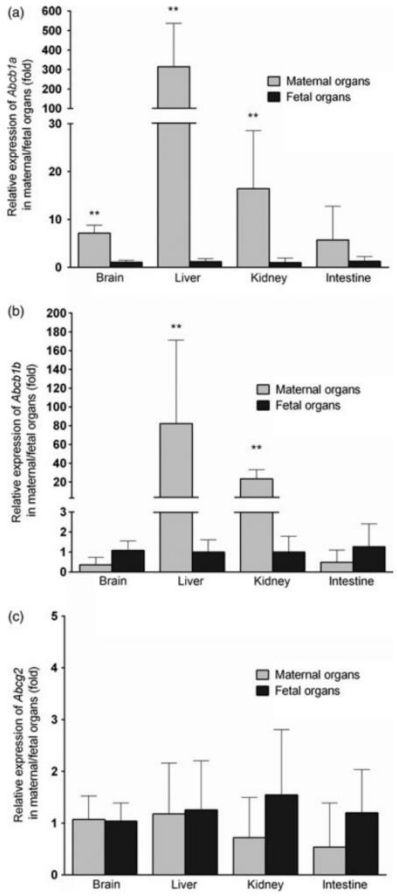


Figure 2 Comparison of *Abcb1a* (a), *Abcb1b* (b) and *Abcg2* (c) expression between maternal and fetal organs analysed in the control group. Brain, liver, kidneys and intestine were collected from pregnant Wistar rats treated with i.m. administered saline from 12th to 21st gestation day. Brain, liver, kidney and intestine of two randomly selected fetuses originated from one dam were collected, homogenized, pooled and taken into analysis as one sample. Due to different expression of reference genes in maternal and fetal organs, obtained data were normalized to total RNA.[30,31] Maternal mRNA expression of target gene is shown as fold change to mRNA expression in appropriate fetal organs. Values represent the means ± SD ($n = 5$). Unpaired nonparametric Mann–Whitney *U*-test was employed to compare mRNA expression in maternal and fetal organs (**$P < 0.01$).

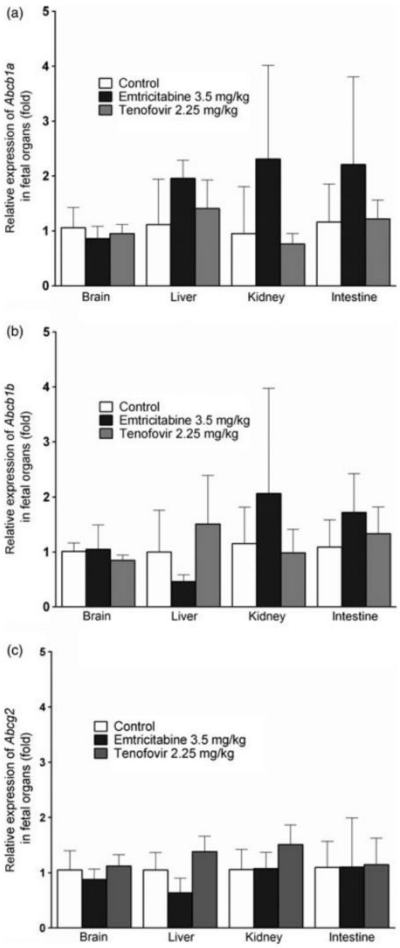


Figure 3 Comparison of *Abcb1a* (a), *Abcb1b* (b) and *Abcg2* (c) expression in the fetal brain, liver, kidneys and intestine from fetuses collected from pregnant Wistar rats exposed to i.m. administration of emtricitabine (3.5 mg/kg/day), tenofovir (2.25 mg/kg/day) or saline (control) from 12th to 21st gestation day. From each dam, organs of two randomly selected fetuses were isolated, homogenized, pooled and taken into analysis as one sample. Data are normalized to two housekeeping genes *B2m* and *Ywhaz* and presented in fold as mean ± SD ($n = 5$ in the control and emtricitabine group; $n = 4$ in the tenofovir group). Statistical significance was examined by unpaired nonparametric Kruskal–Wallis test followed by Dunn's *post hoc* test. *B2m*, beta-2-microglobulin.

means, for example in paediatric clinical trials.[37] Therefore, in our study we took advantage of a living organism,[38] in particular pregnant Wistar rat.[7] The tested compounds were administered i.m. into the back thigh muscle; this type of application ensures precise amount of a drug in the systemic circulation as confirmed by our bioavailability studies (Figure 1). In addition, it (1) avoids stress caused by intragastric gavage that could potentially lead to preterm birth or miscarriage and (2) eliminates the risk of fetus damage by intraperitoneal administration.[39] The amounts of tenofovir (2.25 mg/kg/day) and emtricitabine (3.5 mg/kg/day) were derived from the therapeutic daily doses for HIV-infected adults;[22,40] the dose of tenofovir used in our set-up reflects its pure amount in p.o. prodrug tenofovir disoproxil fumarate.

When comparing expression of these genes between maternal and fetal organs, significantly higher expression of *Abcb1a* was observed in the maternal brain, liver and kidneys (Figure 2a) and higher expression of *Abcb1b* was shown in the maternal kidneys and liver (Figure 2b). These findings are in line with current knowledge on

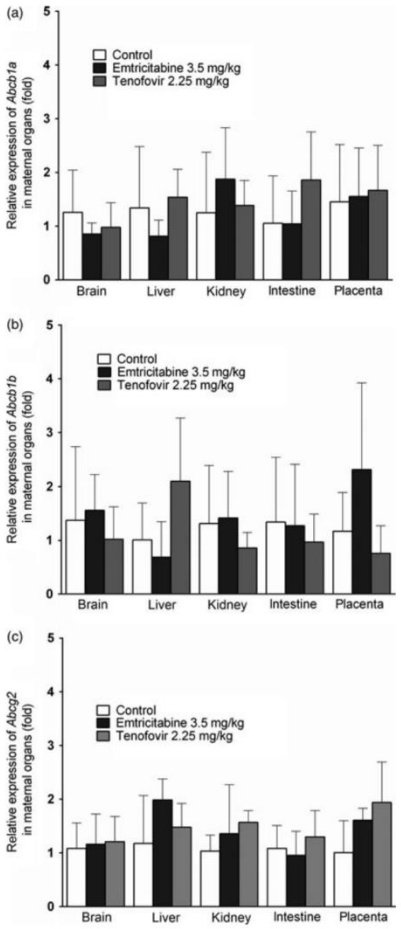
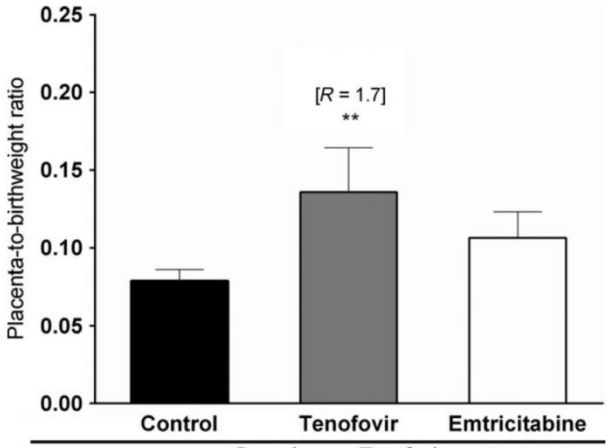


Figure 4 Comparison of *Abcb1a* (a), *Abcb1b* (b) and *Abcg2* (c) expression in the maternal brain, liver, kidneys, intestine and in the placenta isolated from pregnant Wistar rats exposed to emtricitabine (3.5 mg/kg/day), tenofovir (2.25 mg/kg/day) or saline (control) from 12th to 21st gestation day. Data are normalized to two housekeeping genes *B2m* and *Ywhaz* and presented in fold as mean ± SD ($n = 5$ in the control and emtricitabine group; $n = 4$ in the tenofovir group). Statistical significance was examined by unpaired nonparametric Kruskal–Wallis test followed by Dunn's *post hoc* test. *B2m*, beta-2-microglobulin.



	Control	Tenofovir	Emtricitabine
Placental weight (g)	0.46 (±0.04)	0.66 (±0.14)**	0.58 (±0.09)
Birth weight (g)	5.79 (±0.79)	5.06 (±0.90)	5.42 (±0.09)

Figure 5 Monitoring of placental weight, birthweight and analysis of placenta-to-birthweight ratio. Pregnant Wistar rats were exposed to tenofovir (2.25 mg/kg/day), emtricitabine (3.5 mg/kg/day) or saline. On the 22nd gestation day, four fetuses with their corresponding placentas from each animal were taken into analysis. Significantly increased weight of placenta was observed in the tenofovir group, and placenta-to-birthweight ratio was significantly elevated in both tenofovir and emtricitabine group of animals. Data are presented as mean ± SD ($n = 5$ in the control and emtricitabine group; $n = 4$ in the tenofovir group). Statistical significance was examined by unpaired nonparametric Kruskal–Wallis test followed by Dunn's *post hoc* test (**$P < 0.01$). [*R*] value expresses fold increase in the placenta-to-birthweight ratio compared to control group.

ontogeny of *Abcb1a* and *Abcb1b* expression in the rat brain, liver and kidneys.[41] Ontogeny of mechanisms determining absorption, distribution and elimination of many compounds has been previously described as the reason of different pharmacokinetics in children compared with adults.[42–44] In our experimental set-up, fetal expression pattern of *Abcg2* was equal to that observed in mothers (Figure 3c), while de Zwart[37] reported *Abcg2* to have higher expression (ranging from 1.5 to 4.5 fold) in the fetal liver, kidneys and brain at birth when compared with respective organs in adult rats. This discrepancy can be attributed to different type of normalization in our experimental set-up. Opposite to de Zwart,[37] we repeatedly observed different expression of *B2m* and *Ywhaz* genes in the fetal and maternal organs and, therefore, we could not use them as housekeeping genes. Instead, we preferred normalization of expression of target genes to total RNA.[31] Possible drawbacks of this normalization lie in variable rRNA/mRNA ratio between samples, thus not controlling variation inherent in the reverse transcription.[30] On the other hand, it is a valuable method that can be used when changes in expression of housekeeping genes are observed or expected.[31]

In rats exposed to long-term exposure to tenofovir or emtricitabine, we detected insignificant changes in *Abcb1a*, *Abcb1b* and *Abcg2* expression when compared to control group (Figures 3 and 4). Extrapolation of mRNA data to protein expression is not always straightforward as protein levels might be widely affected by various regulatory factors such as sRNAs, ribosomal density and ribosome occupancy, transcriptional and translational activity or, most importantly, by protein half-lives.[45] We, however, believe that our data might be taken as a surrogate for protein expression as both ABCB1 and ABCG2 proteins reveal relatively short half-lives (16 h and approximately 36 h, respectively)[46–48] and both seem to have similar propen-

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sity to mRNA and protein up regulation in induction studies.[49,50]

Interestingly, significantly higher placenta-to-birthweight ratio was observed in tenofovir-treated animals (Figure 5). This parameter has been confirmed as a strong indicator of high blood pressure in childhood,[25] higher cardiovascular morbidity in adults[27] and also correlates with lower Apgar score of newborns.[51] Our findings are in accordance with the data obtained by Gois *et al.*[7] who reported that long-term administration of tenofovir disoproxil fumarate to pregnant rats causes hypertension of offspring.

Conclusions

We evaluated expression patterns of *Abcb1a*, *Abcb1b* and *Abcg2* in the rat placenta and in both maternal and fetal brain, kidneys, liver and intestine. We observed differences between maternal and fetal tissues in expression of *Abcb1a*, *Abcb1b* but not *Abcg2*. In addition, we found that long-term treatment with tenofovir or emtricitabine lacks any significant influence on *Abcb1a*, *Abcb1b* and *Abcg2* mRNA expression in the tested organs. Our findings bring another evidence that tenofovir and emtricitabine have minimal propensity to drug–drug interactions. We propose that neither of these compounds will substantially affect maternal or fetal pharmacokinetics of ABCB1/ABCG2 substrates including antiretrovirals such as abacavir, zidovudine or tenofovir disoproxil fumarate.[10,52,53] On the other hand, long-term administration of tenofovir increases placenta-to-birthweight ratio in rats suggesting its potential to influence developmental programming of the fetus. Our data thus provide new evidence on safety profile of tenofovir and emtricitabine use in pregnancy; however, further research in other mammal species, including humans, is important to fully elucidate this issue.

Declarations

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7.5 Interaction of lamivudine with ABC and SLC transporters *in vitro* and *in vivo*: role in placental transport

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(v recenzním řízení v časopise *Journal of Antimicrobial Chemotherapy,* $IF_{[2015]} – 5.31$)

V rámci této studie, která vznikla díky mezinárodní spolupráci tří pracovišť, jsme se zaměřili na studium interakcí lamivudinu s transportéry ABCB1, ABCG2, ABCC2 a MATE1 s ohledem na transplacentární farmakokinetiku tohoto často užívaného antiretrovirotika. Za tímto účelem jsme použili *in vitro* akumulační a transportní experimenty na MDCKII a MEF3.8 buňkách, *in situ* metodu duálně perfundované potkaní placenty a *ex vivo* experimenty využívající vezikuly z izolované mikrovilózní apikální membrány trofoblastu lidské placenty.

Lamivudin *in vitro* neinteragoval s ABCB1, ABCG2 a ABCC2. Srovnatelné transplacentární clearance v obou směrech rovněž neodhalily aktivní transport lamivudinu zprostředkovaný těmito transportéry. Dále jsme pozorovali, že transport lamivudinu přes potkaní placentu je závislý na pH gradientu, což poukazuje na možnou aktivitu MATE1 a koresponduje s našimi výsledky z *in vitro* experimentů na MDCKII buňkách. Naproti tomu jsme ale neodhalili pH závislost v akumulaci lamivudinu v MVM vezikulech a proto role MATE1 v transplacentární farmakokinetice této látky zůstává neobjasněna a zaslouží si další pozornost.

Interaction of lamivudine with ABC and SLC transporters *in vitro* and *in vivo*: role in placental transport

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Running title: *"Interaction of lamivudine with placental ABC and SLC transporters"*

Key words: antiretroviral therapy, pregnancy, drug transporter, placenta, drug-drug interaction

Abstract

Objectives: Lamivudine is one of the antiretroviral drugs of choice for the prevention of mother-to-child transmission (MTCT) in HIV-positive women. In this study, we investigated the relevance of drug efflux transporters P-gp (MDR1, ABCB1), BCRP (ABCG2), MRP2 (ABCC2) and MATE1 (SLC47A1) for the transmembrane transport and transplacental transfer of lamivudine.

Methods: We employed *in vitro* accumulation and transport experiments on cells (MDCK and MEF3.8) overexpressing drug efflux transporters, *in situ* perfused rat term placenta and vesicular uptake in microvillous plasma membrane (MVM) vesicles isolated from human term placenta.

Results: MATE1 significantly accelerated lamivudine transport in MATE1-expressing MDCK cells, whereas no transporter-driven efflux of lamivudine was observed in MDCK-MDR1, MDCK-MRP2 and MDCK-BCRP monolayers or in MEF3.8-BCRP cells. MATE1-mediated efflux of lamivudine appeared to be a low affinity process (apparent K_m = 4.21 mM, V_{max} = 5.18 nmol/mg protein/min in MDCK-MATE1 cells). Consistent with *in vitro* transport studies, the transplacental clearance of lamivudine was not affected by P-gp, BCRP or MRP2. However, lamivudine transfer across dually perfused rat placenta was pH dependent, indicating possible involvement of MATE1 in the fetal-to-maternal efflux of the drug. Nevertheless, uptake of lamivudine into MVM vesicles did not reveal a significant pH-dependency.

Conclusion: Placental transport of lamivudine does not seem to be affected by P-gp, MRP2 or BCRP. However, a pH-dependent mechanism mediates transport of lamivudine in the fetal-to-maternal direction in rats; we suggest that MATE1 might be responsible for this transport. Further research is needed to fully elucidate the role of MATE1 in placental pharmacokinetics in humans.

Introduction

More than 36 million people are infected with HIV worldwide today.[1] Half of them are women who, if pregnant, carry the risk of transferring the infection to their child *in utero*, at delivery or during breastfeeding. Progress in preventing new HIV infections among children has been dramatic in recent years as the number of children becoming infected with HIV each year dropped by 58 % from 520.000 to less than 240.000 between 2000 and 2014,[1] clearly demonstrating that well-timed antiretroviral prophylaxis can reduce the risk of mother-to-child transmission (MTCT) of HIV. Current guidelines[2, 3] recommend lamivudine ([-]-b-L-2',3'-dideoxy-3'-thiacytidine) as one of the antiretroviral drugs of choice in first line therapy of HIV-positive pregnant women, including first trimester pregnancies. In addition to diminishing the MTCT of HIV-1, lamivudine is also used to decrease the vertical transmission of hepatitis B virus in pregnancy.[4, 5] Fetal exposure to drugs administered to pregnant women is usually considered as potentially toxic and teratogenic for most medications. However, the transplacental passage of antivirals can advantageously ensure infant pre-exposure prophylaxis.[2, 6] Lamivudine is known to cross the placenta, with the fetal-to-maternal area under the concentration-time curve (AUC) reaching 86% in humans.[7] However, detailed knowledge of mechanisms affecting lamivudine transplacental transfer is still lacking.

The transplacental permeability of drugs predominantly depends on their physical-chemical characteristics, which determine the rate of passive diffusion. However, it can also be extensively influenced by transporters expressed in polarized trophoblasts. Several drug efflux transporters of the ABC (ATP-binding cassette) superfamily are functionally expressed on the trophoblast apical microvillous plasma membrane (MVM), diminishing the penetration of drugs from maternal blood to the fetus.[8] Among them, P-glycoprotein (P-gp, MDR1, *ABCB1*), breast cancer resistance protein (BCRP, *ABCG2*) and multidrug resistance-associated protein 2

(MRP2, *ABCC2*) are well confirmed active components of the placental barrier providing fetal protection against potentially toxic compounds, including drugs.[9-11] Besides ABC transport proteins, some members of the SLC (solute carrier) transporter family can further modulate transplacental drug transfer.[8] We have recently described expression of pH-dependent multidrug and toxin extrusion proteins MATE1/Mate1 (*SLC47A1/Slc47a1*) and MATE2/Mate2 (*SLC47A2/Slc47a2)*) in human and rat first trimester and term placentas.[12] Employing a dually perfused rat term placenta model, we showed that Mate1 may represent the efflux component of vectorial drug transfer of metformin and cationic neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) in the fetal-to-maternal direction.[13, 14]

Lamivudine was recently found to interact with MATE1 and its kidney variant MATE2-K.[15] Thus, we hypothesized that MATE1 could also affect the transplacental transport of lamivudine by mediating the efflux from polarized trophoblast cells back to the maternal blood. All three subtypes of human organic cation uptake transporters, OCT1 *(SLC22A1)*, OCT2 *(SLC22A2)* and OCT3 *(SLC22A3)*, were shown to contribute to lamivudine uptake into the cells, with efficacy dependent on kinetic parameters and V_{max}/K_m transport decreasing in the order OCT1 > OCT2 > OCT3.[16, 17] Whereas OCT3 is the predominant OCT transporter in rats,[12, 13] OCT1 and OCT2 were shown to have significant mRNA expression in first trimester as well as term human placenta in our recent study.[12] We therefore employed an *in vitro* vectorial transport system overexpressing OCT1 and MATE1 or OCT2 and MATE1 to investigate the relevance of these transporters for intracellular concentrations and directional, transcellular transport of lamivudine. A few reports have indicated *in vitro* interactions of lamivudine with ABC transporters P-gp[18] and BCRP.[19] However, the *in vivo* relevance of P-gp and BCRP for the transcellular transport of lamivudine remains to be confirmed.

To the best of our knowledge, the possible impact of drug efflux transporters on transplacental pharmacokinetics of lamivudine has not previously been systematically evaluated. In the

present work, we investigated the affinity of lamivudine to efflux transporters MDR1, BCRP, MRP2 and MATE1 and evaluated whether the maternal-to-fetal transfer of lamivudine could be affected by any of these efflux transporters. To address these aims, we performed *in vitro* transport and accumulation assays on cellular monolayers and the *in situ* method of dually perfused rat term placenta. Additionally, we verified the results in human placenta by uptake assay in MVM vesicles.

Methods

Materials and reagents

Lamivudine was kindly provided by the NIH as a part of the NIH AIDS Reagent Program. Radiolabelled lamivudine ([^{3}H]-lamivudine, 21.3 or 5.2 Ci/mmol) was purchased from Moravek Biochemicals (California, USA). Mitoxantrone, ASP^+ and fluorescein isothiocyanate labelled dextran (MW=40 kDa) were obtained from Sigma Aldrich (St. Louis, MO, USA). BCRP inhibitor Ko143 was purchased from Enzo Life Sciences AG (Lausen, Switzerland) and pentobarbital (Nembutal) was purchased from Abbott Laboratories (Abbott Park, Illinois, USA). Cell culture media and sera were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Gibco BRL Life Technologies (Rockville, MD, USA). All other chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). The bicinchoninic acid assay kit (BCA assay) was purchased from Thermo Scientific (Rockford, USA).

Cell lines and cell culture

Madin–Darby canine kidney II (MDCKII) parental cell line and MDCKII cells stably transduced for expression of human transporters P-gp, BCRP or MRP2 further designed as

MDCK-MDR1, MDCK-BCRP or MDCK-MRP2, respectively as well as MEF3.8 spontaneously immortalized embryo fibroblasts derived from triple knockout mdr1a/b$^{-/-}$, mrp1$^{-/-}$ mice transfected with cDNA of human BCRP (MEF3.8-BCRP), were provided by Prof. Piet Borst and Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). All the MDCK and MEF3.8 cell lines were cultured in DMEM medium supplemented with 10% heat-inactivated FBS. Double-transfected MDCKII cell lines stably expressing human OCT1 or OCT2 and MATE1 transporters (MDCK-OCT1-MATE1 and MDCK-OCT2-MATE1), as well as the respective mono-transfected cells (MDCK-OCT1, MDCK-OCT2, MDCK-MATE1) and control vector cells (MDCK-Co), were established and characterized as described previously.[15, 20, 21] Cells were cultured in MEM containing 10% heat-inactivated FBS. All cells used in our experiments were routinely cultivated in antibiotic-free medium and periodically tested for mycoplasma contamination. Stable expression of all inserted human transporters was verified by qRT-PCR and uptake assays with appropriate fluorescence substrates. Cells from passages 10 to 25 were used in all *in vitro* studies.

Animals

Pregnant Wistar rats were purchased from MediTox s.r.o. (Konarovice, Czech Republic) and maintained under 12/12-hours day/night standard conditions with pellets and water provided *ad libitum*. Experiments were carried out on day 21 of gestation. Overnight-fasted rats were anesthetized with pentobarbital (40 mg/kg body weight) administered into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, France, 1986).

Transcellular transport assays in ABC transporter overexpressing MCDK cells

Conventional bi-directional (concentration gradient) assay

Transport assays employing MDCK parental and ABC transporter-expressing MDCK cells were performed on microporous polycarbonate membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, New York) as described previously.[22, 23] MDCK-MDR1, MDCK-BCRP, MDCK-MRP2 or MDCK-parent cells were seeded at a density of 1.5 x 10^6 per insert and cultured 3-4 days until reaching confluency with daily replacement of cell culture medium. Before starting the transport experiment, cells were washed with pre-warmed phosphate buffered saline (PBS) on both the apical and basal sides of monolayers and Opti-MEM with radiolabelled lamivudine was added to either the apical or basolateral compartments. The lowest [^{3}H]-lamivudine concentration used was 8 nM, as this concentration achieved the minimal specific activity required for analysis (0.04 μCi/ml). The experiments were run at 37 °C / 5% CO_2, aliquots of 50 μl were collected at 2, 4 and 6 hours from the opposite compartment and radioactivity was measured by liquid scintillation counting (Tri-Carb 2900 TR Perkin Elmer). At the end of the experiment, leakage of fluorescein isothiocyanate labelled dextran (MW = 40 kDa) was analyzed and accepted at up to a rate of 1% per hour. The percentage of radioactivity appearing in the acceptor compartment relative to the stock solution initially added to the donor compartment was calculated. Transport ratios between basal-to-apical and apical-to-basal translocation after 6 hours' incubation (r_t) were calculated as described earlier[22] by dividing the percentage of drug transported in the basolateral-to-apical direction by the percentage of drug crossing the monolayer in the apical-to-basolateral direction.

Concentration equilibrium method

ABC transporter expressing MDCK cells were cultured as monolayers on cell culture inserts as described above for the concentration gradient method. In these experiments, after washing the confluent monolayers with PBS, the transport assay was initiated by addition of [^{3}H]-lamivudine (8 nM) to both compartments. The experiments were run at 37 °C / 5% CO_2. For drug analysis, aliquots of 50 µl were collected after 6 hours from both compartments and radioactivity was measured by liquid scintillation counting (Tri-Carb 2900 TR Perkin Elmer). Concentration ratios at the end of the assay (r_e) were calculated by dividing the percentage of initial drug concentration in the basolateral compartment to the percentage of initial drug concentration in the apical compartment at the end of experiment (6 hours).

Accumulation experiments on MEF3.8 and MEF3.8-BCRP

To evaluate the possible involvement of BCRP in lamivudine efflux using another cellular model with lower background of endogenous transporters,[24] MEF3.8-BCRP cells were seeded on 24-well plates (1×10^5 cells per well). After reaching 80% confluency, the medium was aspirated, cells were washed with PBS and pre-incubated for 30 min at 37 °C in Opti-MEM medium with or without the BCRP inhibitor Ko143 (1µM). Accumulation of [^{3}H]-lamivudine (100 nM) was performed for 60 min at 37 °C. The concentration of lamivudine was selected to achieve the maximal sensitivity required for detection of interaction with drug transporters while at the same time exceeding the minimal specific activity required for final analysis in cell lysates. The accumulation was stopped by cooling on ice and washing twice with ice-cold PBS. Cells were then lysed in 0.02% SDS. The radioactivity of accumulated [^{3}H]-lamivudine was measured by liquid scintillation counting in cell lysates (Tri-Carb 2900 TR Perkin Elmer). The

protein concentration of the cell lysates was quantified by a BCA assay. Substrate uptake was normalized with respect to protein concentrations of the cell lysate.

Transcellular transport assays in SLC transporter overexpressing MCDK cells

Transport experiments employing mono-transfected MDCK-MATE1, MDCK-OCT1 and MDCK-OCT2 cells, double-transfected MDCK-OCT1-MATE1 and MDCK-OCT2-MATE1 and the empty vector transfected control MDCK-Co cells were performed on Transwell 3402 cell culture inserts (3.0 µm pore size, 24 mm diameter Costar, Corning, NY). For all experiments, 0.5 x 10^6 cells per well were used and incubated for 3 days to confluence in standard cultivation medium MEM (Gibco) + 10% FBS. On the day of experiment, medium was removed from both sides and the cellular monolayer was washed on both sides with pre-warmed PBS. The experiment was started by addition of 0.8 ml HBSS buffer (pH 7.4) into the apical compartment and 0.8 ml of HBSS buffer (pH 7.4) with [^{3}H]-lamivudine to the basolateral compartment. The [^{3}H]-lamivudine concentration used was 100 nM, as this concentration achieved the maximal sensitivity required for detection of interaction with drug transporters and minimal specific activity required for final analysis in cell lysates. In the inhibition experiments with mitoxantrone, the cellular monolayers were pre-incubated with HBSS medium (pH 7.4) containing 2 µM mitoxantrone in both compartments 10 minutes prior to initiation of the transport experiment. The experiments were run at 37 °C / 5% CO_2 for 2 hours. Aliquots of 50 µl were sampled from the apical side at times 0.5, 1 and 2 hours. At the end of the incubation period, the medium was immediately removed and cells were washed twice with ice-cold PBS, then the inserts were excised and the cellular monolayer was dissolved in 0.02% SDS solution. The radioactivity of the collected samples and lysed monolayers was measured by liquid scintillation counting (Tri-Carb 2900 TR Perkin Elmer). Protein concentration in the cell lysates was quantified using a BCA assay. Net transport was obtained by subtraction of the

transport by MDCK-Co cells from that by drug-transporter overexpressing cells. The kinetics graph (transport velocity of lamivudine versus substrate concentration) was fitted using the classic Michaelis-Menten equation, with V_{max} representing the maximal transport velocity (in nmol per mg of protein per minute) and K_m representing the substrate concentration at half-maximal transport velocity (micromolar), in GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, California, USA).

ASP^+ uptake experiments

Uptake experiments were performed using control MDCK-Co and OCT1-, OCT2- and MATE1- monotransfected MDCK cells in order to quantify the inhibitory effect of mitoxantrone to the particular SLC transporters. The aim of these experiments was to show that mitoxantrone could be used as a model inhibitor that could selectively inhibit MATE1 in the subsequent transport studies using OCT-MATE double-transfected cells. Mitoxantrone was chosen as the model inhibitor because it has been shown to preferentially inhibit MATE1 over OCT1 and OCT2 according to the half-maximal inhibitory concentration (IC_{50}) values in other cellular models.[25] MATE1 is a pH-dependent carrier that is able to act as an uptake transporter in experimental settings[26] but mediates efflux in physiological ones. Hence, extracellular alkalinization was used to promote MATE1-mediated uptake.[27, 28] The single SLC transporter-transfected MDCK cells were seeded on a 96-well plate at a density of 45 x 10^3 cells per well and cultivated in standard cultivation medium (MEM + 10% FBS). Twenty-four hours after seeding, uptake experiments with ASP^+, a common fluorescence substrate of OCT1, OCT2 and MATE1, were performed. Cells were washed twice with 100 µl pre-warmed HBSS buffer (pH 7.4). Cell lines containing MATE1 were pre-incubated with 100 µl 20 mM NH_4Cl (pH 7.4) for 30 min. After washing the cells twice with 100 µl pre-warmed HBSS buffer (pH 8.0), solutions of mitoxantrone with 1µM ASP^+ were added and incubated for 20 minutes. After washing the

cells twice with 100 µl pre-warmed HBSS buffer pH 8.0, solutions of mitoxantrone with 1µM ASP^+ were added for 20 minutes. At the end of the incubation period, the medium was removed and cells were rinsed thrice with ice-cold HBSS buffer (pH 7.4). Then, fluorescence was measured at a wavelength of 485 nm for excitation and 585 nm for emission. Substrate uptake was normalized with to the protein concentration of the cell lysate measured by BCA assay.

Dual perfusion of rat term placenta

The method of dually perfused rat term placenta was used as described previously.[29]

Open-circuit perfusion system

An open-circuit perfusion system was employed to study fetal-to-maternal (F→M) and maternal-to-fetal (M→F) lamivudine clearance. [^{3}H]-lamivudine at 12 nM concentration was added to either the maternal (M→F studies) or fetal (F→M studies) reservoirs immediately after successful surgery. After a 5 min stabilization period, sample collection was started (time 0). Fetal effluent was sampled into pre-weighed vials at 5 min interval, the radioactive concentration was measured as above and the transplacental clearance across the time interval was calculated as described below.

Organ clearance was applied to mathematically describe M→F and F→M transport of lamivudine in the open-circuit perfusion system. M→F transplacental clearance (Cl_{mf}) and F→M transplacental clearance (Cl_{fm}) were calculated as described previously.[22, 29] Averaged data recorded at intervals of 10–35 minutes were used for the following calculations. M→F transplacental clearance (Cl_{mf}) normalized to placenta weight was calculated according to Equation. 1:

$$Cl_{\mathrm{mf}} = (C_{\mathrm{fv}}.\, Q_{\mathrm{f}})/(\, C_{\mathrm{ma}}.\, W_{\mathrm{p}}) \qquad (1)$$

where C_{fv} is the drug concentration in the umbilical vein effluent [nmol/l], Q_f is the umbilical flow rate [ml/min], C_{ma} is concentration in the maternal reservoir [nmol/l], and W_p is the wet weight of the placenta [g]. F>M transplacental clearance (Cl_{fm}) was calculated according to Equation 2.

$$Cl_{fm} = (C_{fa} - C_{fv})Q_f/(C_{fa}.W_p) \qquad (2)$$

where C_{fa} is the drug concentration [nmol/l] in the fetal reservoir entering the perfused placenta via the umbilical artery.

Closed-circuit (recirculation) perfusion system

A closed circuit (recirculation) perfusion system was employed to study the effect of pH on the fetal/maternal lamivudine concentration ratio at equilibrium. Both the maternal and fetal sides of the placenta were infused with 9 nM [^{3}H]-lamivudine and after a short-time stabilization period, the fetal perfusate (10 ml) was recirculated for 60 minutes. In the fetal recirculating reservoir, a pH of 7.4 was maintained throughout the experiments, whereas the pH in the maternal reservoir was adjusted by HCl/NaOH to 6.5, 7.4 or 8.5. Samples (250 µl) were collected every 10 minutes from the maternal and fetal reservoirs and the [^{3}H]-lamivudine concentration was measured. This experimental setup ensured a steady concentration on the maternal side of the placenta and enabled investigation of the fetal/maternal ratio; any net transfer of the substrate would imply transfer against a concentration gradient and provide evidence of active transport.

Uptake assay in human placental microvillous plasma membrane vesicles

Human placentas were obtained following written informed consent with the approval of the Faculty hospital Research Ethics Committee (No. 201006 S15P) from uncomplicated pregnancies at term (38–40 weeks of gestation) delivered by Caesarean section in the

Department of Obstetrics and Gynecology University Hospital in Hradec Kralove. MVM vesicles were isolated using Mg^{2+} precipitation and differential centrifugation as described previously. [30] The final MVM pellet was resuspended in an intravesicular buffer (IVB) at two different pH values adjusted by changing the HEPES to Tris ratio (IVB 7.4: 290 mM sucrose, 5 mM HEPES and 5 mM Tris, pH 7.4 or IVB 6.2: 290 mM sucrose, 9.5 mM HEPES and 0.5 mM Tris, pH 6.2), vesiculated by passing 15 times through a 25-gauge needle and stored at -70 °C until use in the uptake experiments. MVM protein concentration was determined using the BCA assay and optimal purity was confirmed by measuring the enrichment of MVM alkaline phosphatase activity compared with the placental homogenate. MVM protein concentration was determined using the BCA assay and purity was confirmed by measuring the enrichment of MVM alkaline phosphatase activity compared with the placental homogenate. The alkaline phosphatase enrichment factor was 21.8 ± 5.6 (mean ± SD, n = 7).

Uptake of [^{3}H]-lamivudine into MVM vesicles was measured at room temperature using rapid vacuum filtration.[31] MVM vesicles (10 mg protein/ml) were equilibrated to room temperature (21–25 °C) prior to uptake. Uptake of [^{3}H]-lamivudine was initiated by mixing 10 µl MVM vesicles with 10 µl 100 nM [^{3}H]-lamivudine in extravesicular buffer (EVB) at pH 7.4 or 8.4 (145 mM KCl, 10 mM Na^+-HEPES/HCl-Tris, pH 7.4 or IVB 8.4). After 1 minute, uptake was stopped by addition of 2 ml ice-cold stop buffer (130 mM NaCl, 10 mM Na_2HPO_4, 4.2 mM KCl, 1.2 mM $MgSO_4$, and 0.75 mM $CaCl_2$, pH 7.4) and filtered through a 0.45 µM mixed cellulose ester filter (MF-Millipore membrane filter HAWP02500) under vacuum. Filters were washed with 10 ml of stop buffer and the filter-associated radioactivity was determined by liquid scintillation counting. No protein controls (replacement of MVM vesicle protein by relevant IVB) were included in parallel to determine tracer binding to the filter, which was subtracted from the total vesicle count.

Statistical analysis

All data were assessed and statistically analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, California, USA). Statistical significance was investigated using Student's *t*-test, the Kruskal-Wallis test or two-way ANOVA followed by Bonferroni's multiple comparison post-test as applicable and described in the figure legends. A P value ≤ 0.05 was taken to be statistically significant. Data are presented as the mean ± standard deviation (SD) or with the 95% confidence interval (CI) where appropriate. Half-maximal inhibitory concentration (IC_{50}) values for mitoxantrone were calculated by non-linear regression using sigmoidal Hill kinetics using GraphPad Prism 6.0 software.

Results

Transcellular transport of lamivudine across ABC transporter expressing MDCK monolayers: bi-directional concentration gradient assay

Transcellular transport of [^{3}H]-lamivudine (8 nM) across the polarized monolayers of MDCK parental and MDR1-, BCRP- and MRP2-overexpressing cells was first studied using the conventional bi-directional (concentration gradient) transport assay. No difference between basal to apical (BA) and apical to basal (AB) transfer of the drug was observed at time points 2, 4 hours and transport ratios (r_t) close to 1 were observed after 6 hours of transport (Table 1). The r_t value obtained in MDCK-BCRP cells (1.25 ± 0.0277) was significantly higher than that measured in MDCK-parent cells (0.923 ± 0.195). However, the cutoff value for BCRP substrates ($r_t \geq 2$) set by the International Transporter Consortium was not reached.[32] These data indicate that lamivudine is not a substrate of P-gp or MRP2, whereas BCRP might to some extent be responsible for acceleration of lamivudine transport in the BA direction.

Transcellular transport of lamivudine across ABC transporter expressing MDCK monolayers: concentration equilibrium assay

We further verified our results obtained in the conventional bi-directional transport assays by measuring lamivudine transport across cellular monolayers in a concentration equilibrium setup; the drug was added to both compartments (apical and basolateral) so that the initial drug concentration was the same on both sides of the monolayer. If the drug was a substrate of apically overexpressed ABC transporter, its concentration should have increased in the apical compartment (and decrease in the basolateral compartment). However, over the 6 hour experiment, we did not observe any significant shift in the lamivudine concentration between either the apical or the basal compartments and the r_e values approached 1 in MDCK-MDR1, MDCK-BCRP, MDCK-MRP2 and MDCK-parent cells (Table 1). Therefore, these data did not indicate significant transport of lamivudine by P-gp, BCRP or MRP2.

Lamivudine accumulation in MEF3.8 cells, MEF3.8-BCRP

To further investigate the relevance of BCRP for lamivudine transport, we employed another cellular model, distinct from MDCK-BCRP cells. Accumulation of lamivudine was analyzed in MEF3.8-BCRP cells and compared to the parental MEF3.8 cell line. After 1 hour's incubation, we did not observe any difference between the intracellular amount of lamivudine present in BCRP expressing and parental cells. Moreover, addition of Ko143, a potent BCRP inhibitor, did not cause any effect on lamivudine accumulation in MEF3.8-BCRP cells (Fig. 1), further confirming that lamivudine is not a BCRP substrate.

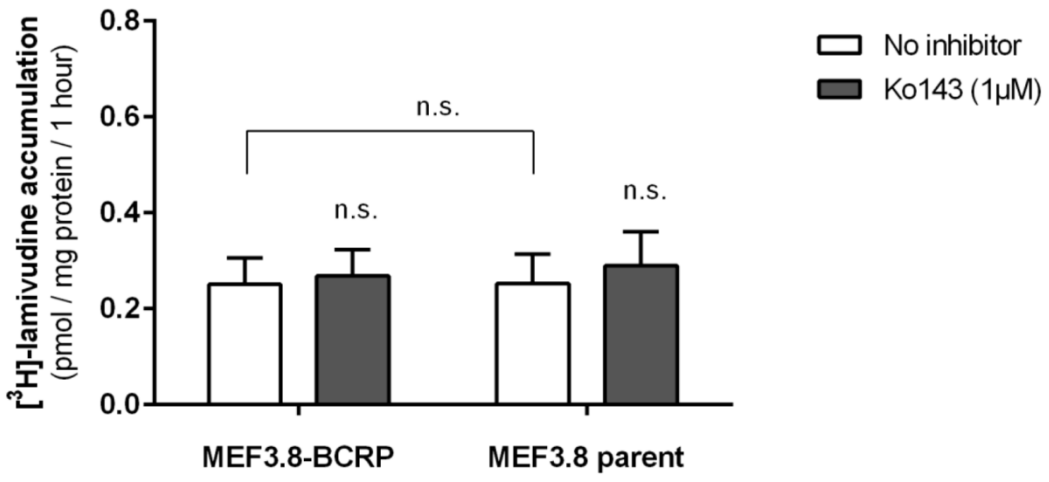

Figure 1. Accumulation of [^{3}H]-lamivudine (100 nM) in MEF3.8-BCRP and control MEF3.8 parental cells in the absence and presence of BCRP specific inhibitor Ko143 (1 μM). Student´s *t*-test was employed to evaluate statistical significance of the difference between drug accumulation in MEF3.8 and MEF3.8-BCRP cells and the effect of Ko143 (n.s. = not significant).

MATE1-mediated transfer of lamivudine across cellular monolayers

After addition of 100 nM lamivudine to the basolateral compartment, all three MATE1 expressing cell lines, i.e. MDCK-MATE1, MDCK-OCT1-MATE1 and MDCK-OCT2-MATE1, showed significantly higher transcellular transfer of [^{3}H]-lamivudine from the basal to the apical compartment compared with MDCK-Co cells (increase to 262.4% , 196.8% and 250.9%, respectively, $P \leq 0.01$, Student´s *t*-test) and their respective non-MATE1 expressing control cells (MDCK-Co, MDCK-OCT1 and MDCK-OCT2, respectively, Fig. 2 A,B,C). The rate of lamivudine transcellular transport was lowest in the MDCK-OCT1/MATE1 monolayers among MATE1-expressing cells, in agreement with the lowest gene expression of *hMATE1* in this cell line shown by qRT-PCR (data not shown).

Intracellular concentrations of lamivudine in the monolayers of MDCK–MATE1, MDCK-OCT1-MATE1 and MDCK-OCT2-MATE1 cells were significantly lower than in the respective MATE1-free control cell lines (decrease to 7.31%, 62.1% and 24.6% compared to MDCK-Co, MDCK-OCT1 and MDCK-OCT2, respectively, $P < 0.001$, Student´s *t*-test, Fig. 2 D,E,F). As expected, monolayers of MDCK-OCT1 and MDCK-OCT2 in the transport experiment accumulated significantly higher amount of lamivudine compared to MDCK-Co cells ($P < 0.05$, Student´s *t*-test). Interestingly, the transcellular transfer of lamivudine across MDCK-MATE1 cells was not significantly different from that in double-transfected MDCK-OCT2-MATE1 cells that express the same level of *hMATE1* mRNA (data not shown) but accumulated a higher amount of lamivudine due to OCT2-mediated uptake (Fig 2 D, F).

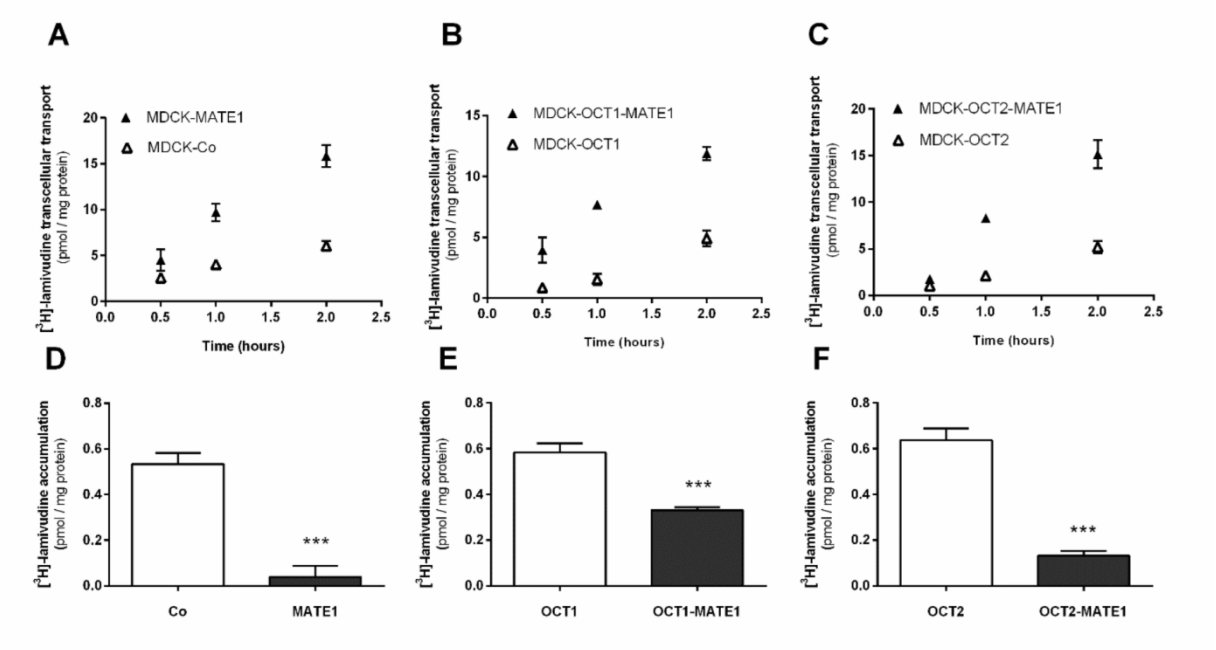

Figure 2. Transport of lamivudine in single-transfected MDCK cells overexpressing OCT1, OCT2 and MATE1, double-transfected MDCK-cells overexpressing OCT1 or OCT2 and MATE1 (MDCK-OCT1-MATE1, MDCK-OCT2-MATE1) and vector control cells MDCK-Co. Cells were seeded on Transwell semipermeable supports dividing a basal compartment and

an apical compartment. Lamivudine (100 nM) was added to the basal compartment and sampled at time points 0.5, 1 and 2 h from the apical side of monolayers (A, B, C). Intracellular accumulation of lamivudine in the monolayers was determined in cell lysates at the end of the experiment (D, E, F). Data were analyzed by Student´s *t*-test (***$P < 0.001$ vs. respective control, Co, OCT1 or OCT2) and are shown as means ± SD ($n \geq 3$).

The apparent affinity of lamivudine to the MATE1 transporter was further evaluated by transport assays in MDCK-MATE1 monolayers using lamivudine concentrations ranging from 100 nM to 10 mM. The K_m value for transfer of lamivudine across MDCK-MATE1 monolayers was 4213 ± 558.2 µM (Fig. 3), indicating that MATE1-mediated transfer of lamivudine is a low affinity process.

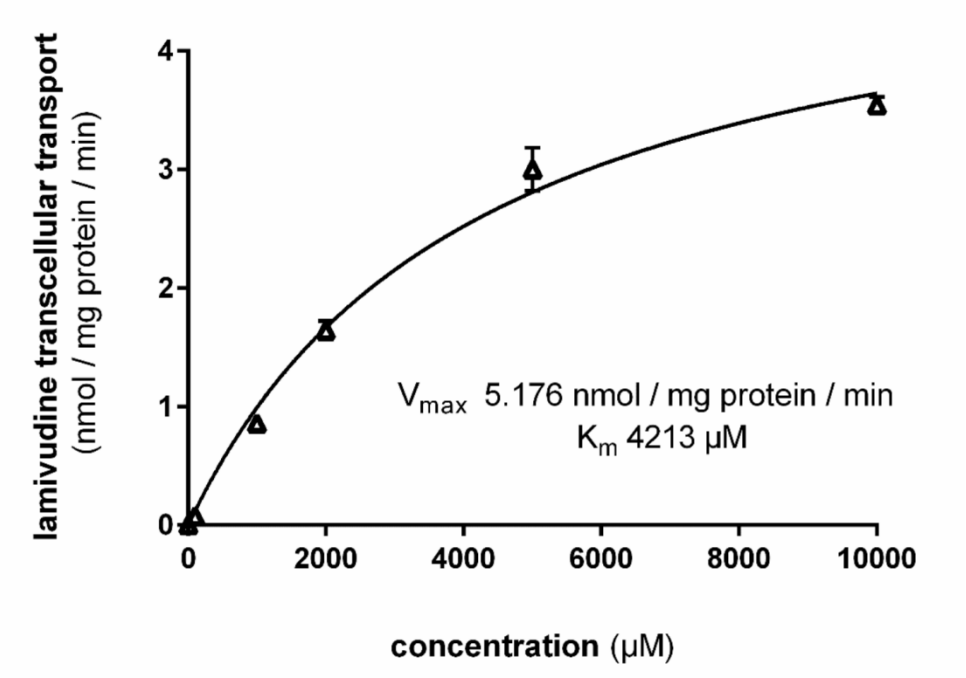

Figure 3. Concentration-dependent net transcellular transport of lamivudine by MATE1**.** Basolateral-to-apical transport of lamivudine across MDCK-MATE1 and MDCK-Co cells cultured as monolayers on Transwell membranes was investigated for increasing concentrations of non-radiolabelled lamivudine (1 x 10^{-4}; 1 x 10^{-3}; 0.01; 0.1; 1.0; 2.0; 5.0 and 10 mM) with

addition of tracer [^{3}H]-lamivudine (16.7 nM) applied to the basolateral compartment. The transcellular transport of lamivudine across MDCK-Co monolayers was subtracted from that in MDCK-MATE1 cells at each concentration point. Kinetic parameters (K_m and V_{max}) were estimated by fitting MATE-specific transport rates to a Michaelis-Menten non-linear equation. Data (nmol/mg protein/min) represent the mean ± SD from three independent experiments.

Mitoxantrone-mediated MATE1 inhibition of lamivudine transport

To further test the contribution of MATE1 to the transcellular transfer of lamivudine, a potent inhibitor of MATE1, mitoxantrone, was employed. First, ASP^+ uptake was investigated in MDCK-OCT1, MDCK-OCT2 and MDCK-MATE1 cells to assess the inhibitory potency of mitoxantrone to the different SLC transporters (accumulation buffer of pH 7.4 was used in both OCT-expressing cells, whereas pH 8.4 was used for the MDCK-MATE1 cells to reverse the direction of the ASP^+ transport to uptake). When comparing IC_{50} values, the observed selectivity for MATE1 inhibition was 4.8 and 9.1 times higher than that for OCT1 and OCT2 ($P < 0.001$), respectively (Fig. 4A). Based on these results and the dose dependency inhibition curve of ASP^+ uptake, 2 µM mitoxantrone was chosen for the subsequent transport assay to predominantly inhibit MATE1 over OCTs in MATE1/OCT transporters expressing cellular monolayers (Fig. 4B,C).

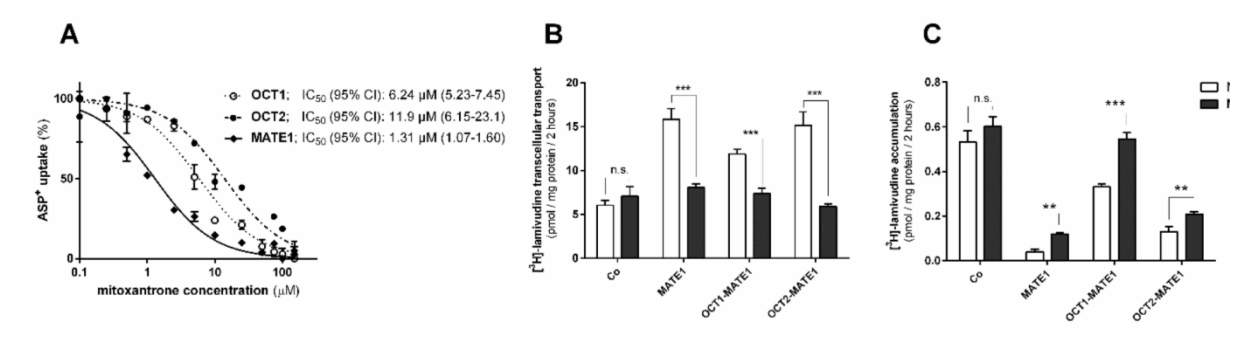

Figure 4. Inhibitory effect of mitoxantrone on lamivudine transport by MATE1. A: IC_{50} values reflecting inhibition of ASP^+ uptake into OCT1-, OCT2-, and MATE1-expressing MDCK cells by mitoxantrone. The IC_{50} values with 95% confidential interval were calculated from three independent measurements. B, C: Effect of 2 µM mitoxantrone on transcellular transport (B) and intracellular accumulation (C) of lamivudine (100 nM) in monolayers of MATE1-expressing and control cells. Data were analyzed by two-way ANOVA with multiple comparisons (**$P < 0.01$, ***$P < 0.001$ vs. respective non-inhibited controls) and are shown as mean ± SD ($n \geq 3$).

Addition of mitoxantrone (2 µM) to the cellular monolayers reduced the basolateral to apical transport of lamivudine in all the MATE1 expressing cells (MDCK-MATE1, MDCK-OCT1-MATE1 and MDCK-OCT2-MATE1) to the level of MDCK-Co control cell line ($P < 0.001$, two-way ANOVA, Fig. 4B) and showed no statistically significant difference in lamivudine transport among the mitoxantrone inhibited cell lines. No effect of mitoxantrone on the transcellular transport across MDCK-OCT1 and MDCK-OCT2 monolayers was observed (data not shown). Consistent with these data, addition of mitoxantrone significantly increased lamivudine accumulation in the monolayers of MATE1-, OCT1-MATE1- and OCT2-MATE1 expressing cells ($P < 0.01$, two-way ANOVA), whereas no increase was observed in MDCK-Co cells (Fig. 4C).

Open circuit perfusion experiments: transplacental clearances of lamivudine in the M→F and F→M direction

The maternal or fetal side of the placenta was perfused with [^{3}H]-lamivudine at a concentration of 12 nM. The ratio between F→M and M→F clearances was 1.8, indicating possible active transplacental transport of lamivudine from the fetal to maternal side, but the differences between M→F and F→M clearances did not reach statistical significance ($P = 0.096$, Student´s *t*-test; Fig. 6). Less than 1% of the lamivudine dose was detected in the placenta after the perfusion experiments, suggesting limited tissue binding or accumulation in trophoblasts and negligible effect on the clearance calculation.

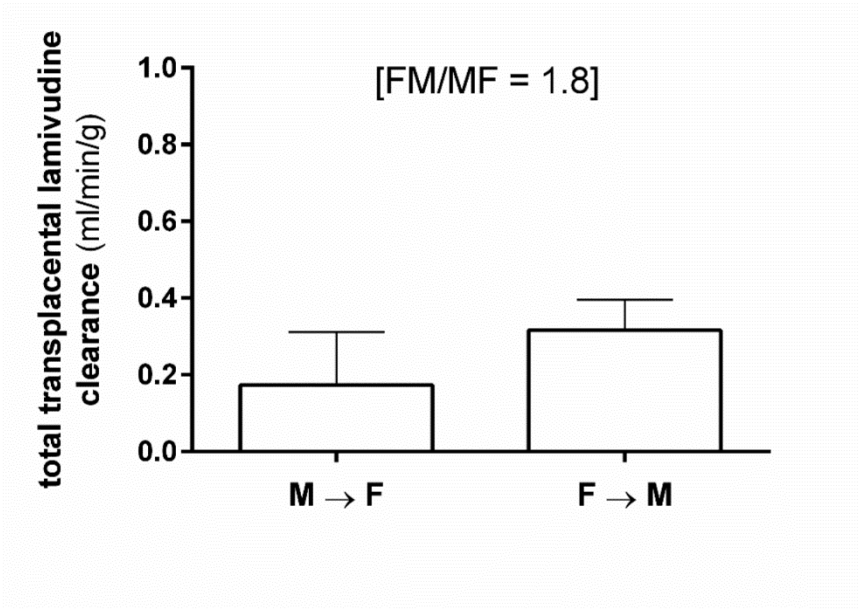

Figure 5. Lamivudine transport across dually perfused rat term placenta in the M→F and F→M directions. [^{3}H]-Lamivudine at 12 nM concentration was added to either the maternal or fetal compartment and the tracer concentration was measured in the fetal venous outflow. Total transplacental clearance was calculated by eq. 1 and 2 (see *Materials and Methods*). No

significant differences between clearances were observed (number in brackets shows the ratio between the F→M and M→F clearances). Data are presented as means ± SD ($n \geq 3$).

Closed circuit perfusion experiments: effect of pH on lamivudine transport across the placenta

To further study lamivudine transplacental transport, both sides of the placenta were perfused with the same concentration of [^{3}H]-lamivudine (12 nM) in a closed circuit experimental setup. We observed only a slight decrease of lamivudine concentration in the fetal perfusate, achieving 95.3 ± 2.46 % of the initial concentrations over 60 min perfusion. This decline might indicate a small contribution of an active transport against the concentration gradient from the fetal to the maternal side of the placenta. To study the effect of pH on lamivudine fetal-to-maternal transport, which could reflect involvement of MATE1-mediated efflux, the pH in the maternal reservoir was set to 6.5 or 8.5, while the pH in the fetal reservoir was set to 7.4. The fetal lamivudine concentration changed significantly between the experiments with pH 6.5 and 8.5 (Fig. 6), suggesting involvement of proton-dependent transport of lamivudine across the placenta.

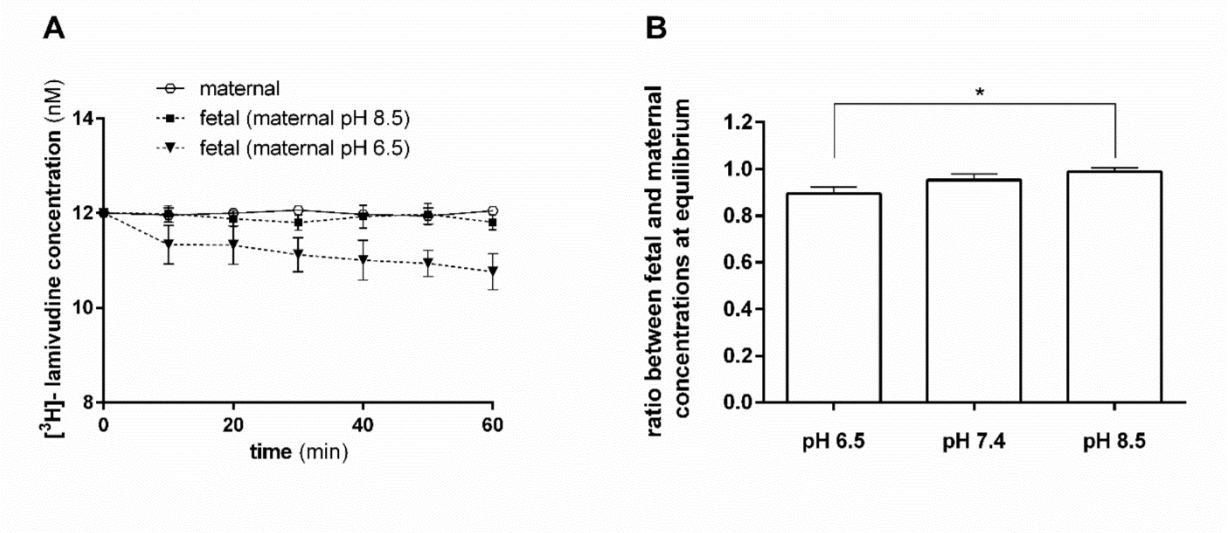

Figure 6. Effect of maternal pH on elimination of lamivudine from the fetal circulation. In the closed-circuit perfusion setup, both the fetal and maternal sides of the placenta were simultaneously infused with 12 nM [^{3}H]-lamivudine. The fetal pH was set to 7.4, whereas the pH in the maternal reservoir was set to 6.5, 7.4 or 8.5. The fetal perfusate was recirculated for 60 min and then, fetal and maternal concentrations of lamivudine were compared. A: Lamivudine fetal concentration over 60 min perfusion at pH 6.5 and 8.5 applied on the maternal site. B: Final ratio between fetal and maternal concentrations at equilibrium showing statistically significant difference between ratios calculated for perfusions at pH 6.5 and pH 8.5 ($P < 0.05$; Kruskal-Wallis test), suggesting involvement of a proton-cation antiporter system in lamivudine transplacental transport. Data are presented as means ± SD ($n \geq 3$).

Uptake of [^{3}H]-lamivudine into human placenta MVM vesicles: effect of pH

To determine the relevance of MATE1-mediated transport for the transplacental transfer of lamivudine in human placenta, and, more specifically, MATE1 involvement in the transport of lamivudine across the MVM of human placenta, uptake of lamivudine into MVM vesicles isolated from human term placenta of uncomplicated pregnancies was measured. The

lamivudine accumulation into the vesicles tended to be stimulated by a higher extravesicular pH. However, the magnitude of the increase in response to the imposed outwardly directed proton gradient was rather variable between MVM vesicle isolates and did not reach statistical significance (Fig. 7).

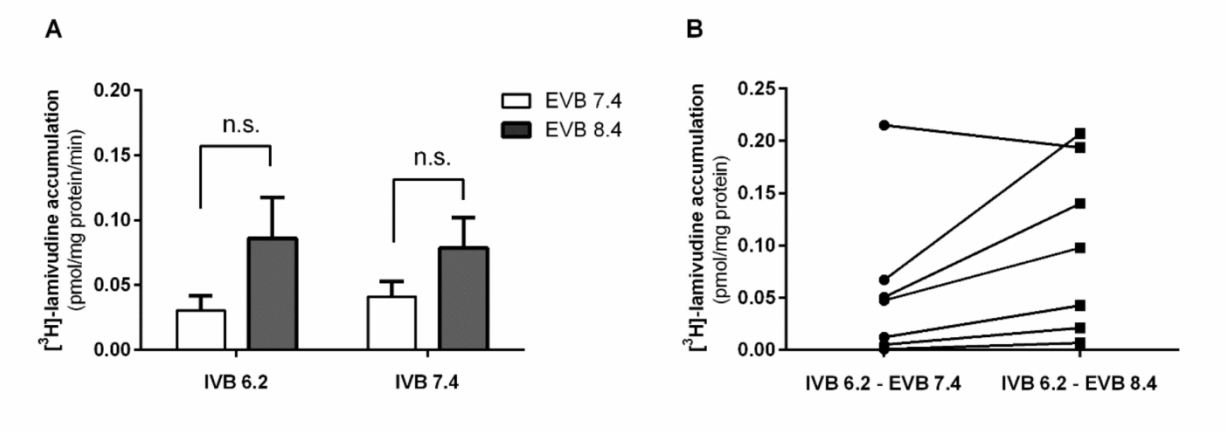

Figure 7. Effect of H^+ gradient on [^{3}H]-lamivudine uptake by MVM vesicles from human term placentas. MVM vesicles were prepared in intravesicular buffer (IVB) at pH 6.2 or 7.4. A: One minute uptake of [^{3}H]-lamivudine was examined in extravesicular buffer (EVB) containing 100 nM [^{3}H]-lamivudine at pH 7.4 or 8.4. B: Paired measurements for pH 6.2 IVB MVM vesicles showing stimulation of [^{3}H]-lamivudine uptake in 6 of the 7 isolates in the presence of an increased pH gradient (change = 205 ± 141%, mean ± SD, n=7; P=0.075, paired t-test). Data are presented as means ± SD of experiments obtained from 5-7 placentas.

Discussion

Lamivudine is considered a first line antiretroviral drug to prevent MTCT in HIV-positive pregnant women.[2] The concentrations of lamivudine found in cord blood at delivery have been reported to reach maternal levels, suggesting that the drug can freely cross the placenta by

passive diffusion.[33, 34] Nevertheless, a fetal-to-maternal area under the concentration-time curve (AUC) ratio of 0.86[7] indicates that transplacental crossing of lamivudine is not entirely passive. One explanation is that a fetal-to-maternal directed transport mechanism is involved which diminishes the transplacental passage of lamivudine from mother to fetus. Such information would be of considerable importance for optimizing the pharmacotherapy of pregnant women because placental drug transporters could be involved in pharmacokinetic drug-drug interactions (DDI) affecting fetal drug exposure of concomitantly administered drugs and leading to impaired treatment outcome or adverse effects.[8, 35] This issue is of particular importance in anti-HIV therapy, where combination of two or more antiretroviral drugs is recommended.[2]

Several drug efflux transporters are functionally expressed in the apical microvillous plasma membrane of human syncytiotrophoblast. Among these are the ATP-dependent transporters, P-gp (MDR1, ABCB1), BCRP (ABCG2) and MRP2 (ABCC2), which actively efflux potentially toxic compounds, including drugs, in the fetal-to-maternal direction, and are therefore considered important fetus-protecting components of the placental barrier.[9-11] We have recently shown that the transplacental transfer of three antiretroviral drugs, tenofovir disoproxil fumarate (TDF)[22], abacavir[23] and zidovudine[36] are affected by drug efflux transporters P-gp and BCRP, suggesting that pharmacokinetic DDI might occur when these drugs are combined with other ABC transporter-interacting drugs during antiretroviral therapy. In the present project, we aimed to assess whether lamivudine is a substrate of placental drug transport proteins and address whether transplacental transfer of lamivudine is affected by drug efflux transporters.

Interaction of lamivudine with P-gp was previously suggested by de Souza et al.,[18] who used a transport assay in P-gp expressing monolayers. However, based on the lack of interaction of lamivudine with a potent P-gp inhibitor (GG 918), the authors concluded that P-gp does not significantly affect the transport of lamivudine.[18] Correspondingly, we did not observe any P-

gp-accelerated transport of lamivudine (8 nM) across the MDCK-MDR1 monolayers in conventional bi-directional concentration gradient assay or equilibrium transport assay, confirming the lack of relevant P-gp involvement in lamivudine transport.

Transport of lamivudine by BCRP was suggested by Kim et al., who observed decreased lamivudine uptake in MDCK-BCRP cells and saturable lamivudine transport across BCRP expressing monolayers.[19] Nevertheless, functionally relevant polymorphic variants of BCRP had no effect on lamivudine disposition in healthy volunteers, indicating that BCRP is unlikely to make a relevant contribution to lamivudine pharmacokinetics. To further address this issue, we performed transport studies with a low lamivudine concentration in MDCK-BCRP cells using both a concentration gradient and concentration equilibrium setup. The ratio between BA and AB transport observed in the bi-directional concentration gradient assay did not reach the value of 2, which is considered by the International Transporter Consortium guidelines as a cut-off level for classifying a drug as a transporter substrate.[32] Nevertheless, because the lamivudine transport ratio (r_t) in MCDK-BCRP significantly exceeded that in the parental MDCK cells (Table 1), we hypothesized that lamivudine might be a substrate of BCRP, albeit with very low affinity. Therefore, to verify this suggestion, we employed the MDCK transport assays in the concentration equilibrium setup and accumulation assays in MEF3.8–BCRP cells, another cellular model used for the identification of drugs interacting with BCRP.[37] However, none of these approaches revealed BCRP-mediated efflux of lamivudine (Table 1, Fig. 1), confirming our findings in the transporter assays and questioning the transport of lamivudine by BCRP observed by Kim et al.[19] Similarly to the results in MDCK-MDR1 and MDCK-BCRP cells, no active transporter-driven transfer of lamivudine was observed in MDCK-MRP2 cells, showing that lamivudine is not significantly transported by any of the three main placental ABC transporters.

In situ dually perfused rat term placenta represents a valuable alternative model to study placental pharmacology and physiology. Using this approach, we have previously identified ABC transporter-mediated placental passage of several compounds, including antiretrovirals.[22, 23, 29, 38] In the present study, we showed that the fetal-to-maternal clearance of lamivudine was not significantly higher than the maternal-to-fetal clearance (Fig. 5), in agreement with the above-discussed *in vitro* transport experiments and indicating no, or only negligible, involvement of active fetus-to-mother directed efflux of lamivudine.

MATE1 (*SLC47A1*), expressed in the apical membrane of polarized cells, is an H^+ - exchanger known to ensure the efflux of substrates that enter the cells via organic cation transporters (OCTs, *SLC22A*) located in the basolateral membrane.[39] This OCT-MATE1 excretory pathway is typical for the kidneys and liver. Nevertheless, our recent studies have indicated that this vectorial transport mechanism might also be relevant for the placenta.[12, 13] All three OCTs have been detected in both rat and human placentas, with OCT3 being the predominant variant in rat placenta[12, 13].[11, 12] Whereas Sato et al. failed to detect OCT1 and OCT2 mRNA in human placenta using end-point RT-PCR,[40] Lee et al. detected all three OCT isoforms, with highest but variable expression for OCT3,[41] and significant expression of OCT1 and OCT2 mRNA was found in human first trimester and term placentas by our group.[12] Lamivudine has recently been shown to be a substrate of human MATE1 and the kidney-specific MATE2-K transporters; MATEs-mediated pH-dependent efflux was suggested to contribute to renal tubular excretion of lamivudine.[15] This antiretroviral has also been shown to be a substrate of all three subtypes of human organic cation transporter, with efficacy dependent on kinetic parameters and V_{max}/K_m ratio decreasing in the following order: OCT1 > OCT2 > OCT3.[16, 17] In the present study, we evaluated the affinity of lamivudine to the MATE1 transporter and addressed in detail the role of OCTs in the transcellular transfer of lamivudine using double-transfected MDCK-OCT1-MATE1 and MDCK-OCT2/MATE2 cell lines and relevant mono-transfected and vector

control MDCK cells. By applying low concentrations of lamivudine to increase the sensitivity of the assay, we confirmed that MATE1 significantly increases the transcellular passage of lamivudine whilst decreasing intracellular accumulation of the drug (Fig. 2). Monolayers of OCT1- and OCT2-single-transfected cells accumulated higher levels of lamivudine but retained a similar transcellular transport efficacy to that in vector control cells, confirming that OCT transporters affect the influx of lamivudine into the cells. In agreement with previous observations with 10 μM lamivudine,[15] lamivudine transfer across MDCK-OCT2-MATE1 monolayers did not differ significantly from that in MDCK-MATE1 cells.[15] Mitoxantrone used at a concentration preferentially inhibiting MATE1 over OCT1 and OCT2 (2 μM) significantly decreased the lamivudine transcellular transfer in all the MATE1 expressing cells to the level of control cells but increased the cellular accumulation in all the MATE1-expressing cell lines (Fig. 3). Our data thereby suggest that lamivudine transcellular transfer is controlled mainly by MATE1-mediated efflux and is not significantly affected by OCT-mediated uptake.

Kinetic analysis of the MATE1-mediated transport of lamivudine revealed a rather low affinity and transport capacity of MATE1 to the antiretroviral (K_m = 4.21 mM, V_{max} = 5.18 nmol/mg protein/min). The transport efficacies of OCT1 and OCT2 transporters to lamivudine have been reported as $V_{max}/K_{m\ (OCT1)}$ = 8.03 (or 8.0 μl/mg protein/min)[16] and $V_{max}/K_{m\ (OCT2)}$ = 4.1 (or 4.4 μl/mg protein/min)[17], respectively, exceeding the efficacy of MATE1 in the transport of lamivudine (V_{max}/K_m = 1.23 μl/mg protein/min). We therefore suggest that MATE1-mediated efflux might be the rate-limiting step in the transcellular transfer of lamivudine, which is consistent with the results of our transport studies showing that the transport rate of lamivudine across MDCK-MATE1 cells did not differ from that across MDCK-OCT2-MATE1 cells (Results 3.1). Nevertheless, the *in vitro* determined K_m values do not necessary preclude kinetic impacts of the transporter *in vivo*. For instance, some drugs of high K_m values determined *in vitro* play a significant role in MATE1 excretory pathways *in vivo*.[42]

Moreover, the concentration of half maximal velocity lamivudine transport by MATE1 is about three orders of magnitude higher than the maximal therapeutic concentration (≈ 3-11 μM) achieved in the plasma of pregnant women,[34, 43] thus making saturation of MATE1 *in vivo* unlikely.

The involvement of pH-dependent transport in the transplacental pharmacokinetics of lamivudine was further evaluated *in situ* by employing dually perfused rat term placenta. Whereas expression of *Mate1* appears to be absent in murine placenta[44, 45] abundant placental expression has been found in rats.[13, 41, 46] Using the technique of dually perfused rat term placenta, we recently demonstrated that MATE1 is able to mediate the pH-dependent transfer of MPP^{+}[13] and metformin[14] in the fetal-to-maternal direction, thereby decreasing fetal exposure to both substrates. In contrast to rat, human placenta seems to express only low and variable levels of MATE1 mRNA.[13, 41] However, a functional contribution of this transporter to the transplacental transfer of drugs cannot be excluded. We observed that decreasing the maternal pH increased the fetal-to-maternal transfer of lamivudine in the closed circuit setup, indicating involvement of a pH-dependent transport mechanism on the maternal facing side of trophoblasts in the transplacental transfer of lamivudine. The maternal-fetal interface does not offer such a steep pH gradient as proximal tubules in the kidney. Nevertheless, it cannot be excluded that MATE1-mediated transport is linked to other H^{+} transferring transporter(s) providing a sufficient H^{+} ion gradient is established to drive the efflux.

To address the relevance of these findings in humans, we additionally evaluated the pH-dependent transport of lamivudine directly on isolated microvillous plasma membrane (MVM) vesicles of human term placental trophoblasts. To promote pH-dependent uptake, vesicles of different intravesicular pH (6.2 or 7.4) were exposed to lamivudine containing extravesicular buffer of increasing pH (7.4 or 8.4), thereby creating an outwardly directed H^{+} gradient of increasing magnitude. Our data showed a tendency for intravesicular lamivudine accumulation,

stimulated by a lower intravesicular pH and higher extravesicular pH. However, the data were variable and failed to achieve statistical significance, which may, in part, reflect the variable gene expression encoding MATE1 in human placenta.[13, 41] Additionally, it is possible that temporal dissipation of the H^+ gradient across the MVM plasma membrane contributed to the variability in the capacity of MATE1 to accumulate lamivudine as substrate,[30] although in a majority of MVM isolates, this was stimulated by a 2.2 pH unit (pH 6.2-pH 8.4) transmembrane gradient. The imposition of a steeper transmembrane H^+ gradient across the MVM plasma membrane may help clarify this further. Moreover, the low expression of MATE in human placenta at term in comparison to the first trimester[12] allows us to hypothesize that the MATE1-mediated transplacental transport of lamivudine might be of higher importance in earlier phases of pregnancy and could be responsible for eventual DDI with other MATE1-interacting drugs. However, the uptake of lamivudine across MVM appears to be sensitive to the imposed H^+ gradient, suggesting that MATE1-mediated H^+/ lamivudine exchange may contribute to the transplacental transfer of lamivudine across human placenta.

To conclude, we have shown here that P-gp, MRP2 and BCRP do not affect the transplacental transfer of lamivudine, making the risk of pharmacokinetic DDI between lamivudine and other antiretroviral substrates of the ABC transporters in the placenta unlikely. On the other hand, we demonstrated a low affinity efflux of lamivudine by MATE1, which seems to act independently of the OCT-mediated cellular uptake of lamivudine. We further showed that a pH-dependent mechanism mediates transport of lamivudine in the fetal-to-maternal direction in rats and concluded that MATE1 might at least be partly responsible for this transport. However, further research is needed to address the role MATE1 may play in human placenta during pregnancy and to elucidate the risk of MATE1-mediated DDI in the transplacental pharmacokinetics of lamivudine in humans.

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FM holds a minor share of stock in Novartis, received research funding from Sanofi-Aventis Deutschland and is an employee of Boehringer Ingelheim Pharma GmbH & Co. KG.

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Table 1. Lamivudine transport across monolayers of MDCK parent and human MDR1-, BCRP- and MRP2-transporter expressing MDCK cells in bi-directional and concentration equilibrium assays.

	Bi-directional assay	**Concentration equilibrium assay**
Cell line	Transport ratio (r_t) (basal-to-apical/apical-to-basal)	Concentration ratio (r_e) (apical-to-basal)
MDCK parent	0.923 ± 0.195	1.02 ± 0.0180
MDCK-MDR1	0.930 ± 0.140	1.01 ± 0.0142
MDCK-BCRP	1.25*± 0.0277	1.03 ± 0.00793
MDCK-MRP2	1.03 ± 0.131	1.00 ± 0.00428

Cells were seeded on Transwell semipermeable supports and grown as monolayers dividing, similarly to a polarized trophoblast layer, the basal compartment (corresponding to fetal side) and apical compartments (corresponding to maternal compartment). The ratios for translocation of lamivudine (8 nM) in monolayers of ABC-transporter expressing cells to MDCK parent control cells after 6 h are shown. Data are presented as means ± SD of ratios obtained in at least four independent experiments ($n \geq 4$). In the bi-directional concentration gradient assay, transport ratios (r_t) for lamivudine translocation in basal-to-apical and apical-to-basal directions were calculated; the concentration ratio (r_e) in the concentration equilibrium assay was calculated from final lamivudine concentrations in apical and basal compartments. Student´s *t*-test was employed to compare the ratios of particular ABC-transporter expressing cells to the respective ratio in MDCK parent control cells and results were considered statistically significant when $P \leq 0.05$(*).

8 Závěr

Nezastupitelnou úlohu v terapii HIV infekce u těhotných žen představuje antiretrovirální léčba, která je doporučovaná po celou dobu těhotenství za účelem minimalizace rizika přenosu viru na dítě. Pro optimalizaci terapie a nalezení vhodné kombinace léčiv, která zajistí léčebný a profylaktický efekt jak v krvi matky, tak i v cirkulaci plodu a zároveň bude pro oba jedince bezpečná, je nezbytné využít znalostí mechanismů přestupu léčiv přes placentární bariéru.

V současnosti je známo, že na distribuci léčiv mezi matkou a plodem se podílí placentární transportéry, u kterých bylo prokázáno, že umožňují či naopak limitují přestup řady látek přes placentu. Mezi doposud nejlépe prozkoumané placentární transportéry se řadí zástupci z rodiny ABC transportérů, jmenovitě jsou to ABCB1, ABCG2 a ABCC2. Tyto transportéry jsou lokalizovány na apikální straně syncytiotrofoblastu, kde aktivně pumpují své substráty zpět do krve matky. U mnohých antiretrovirotik byly popsány interakce s ABC transportéry, kdy tato léčiva vystupovala jako substráty daných transportérů, jejich inhibitory či induktory. Vzhledem k tomu, že léčiva jsou podávána ve formě různých kombinačních koktejlů, znalosti týkající se vzájemných interakcí léčiv s placentárními transportéry tak nabývají klinického významu.

Jedním z cílů této práce bylo přispět k objasnění, zda hlavní ABC placentární transportéry ovlivňují transplacentární farmakokinetiku zvolených léčiv. Antiretrovirotika, která byla ke studiu této problematiky vybrána, patří v současnosti mezi nejužívanější léčiva v terapii HIV infekce u těhotných žen. Do studie jsme zařadili léčiva s dlouhou klinickou praxí, jejichž interakce s ABC transportéry již byly studovány nicméně s nejednoznačným výsledkem (AZT, lamivudin), ale i novější léčiva, u nichž informace týkající se jejich potenciálu interagovat s placentárními transportéry částečně nebo úplně chybí (TFV, TDF, emtricitabin).

K objasnění cílů naší práce jsme využili řadu metodik, které jsme kombinovali tak, abychom získali komplexní obraz o transplacentární kinetice testovaných léčiv. Pro prvotní screening jsme se řídili doporučením lékových agentur jako je americká FDA nebo evropská EMA, které doporučují při studiu lékových interakcí používat transportní experimenty na buněčné linii transdukované lidským transportérem. Podstatou této

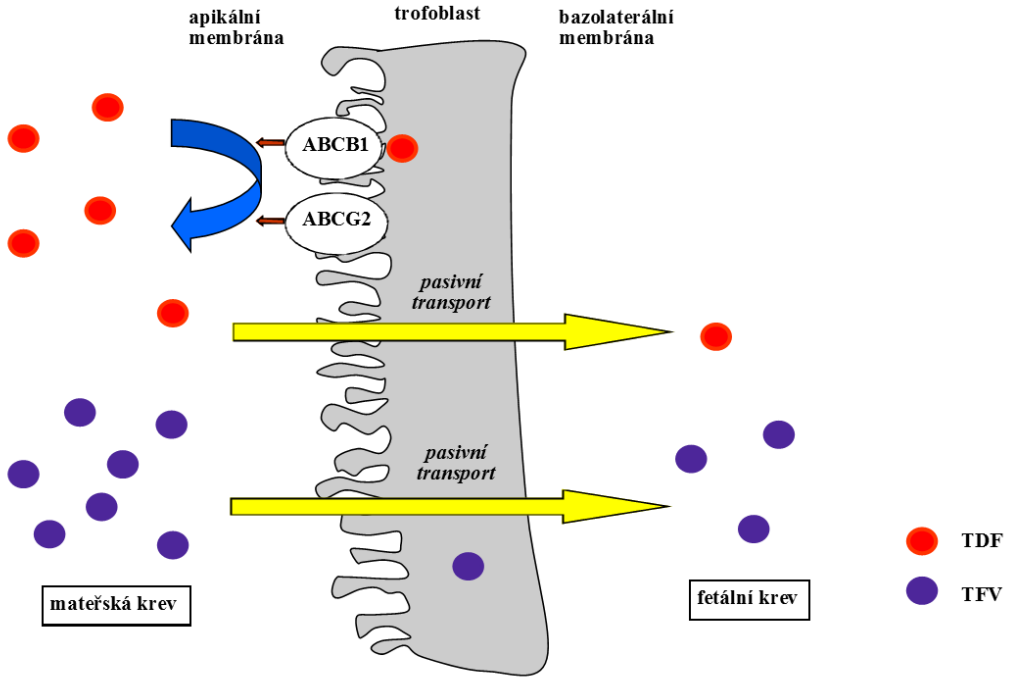
metody je vyhodnocení transportu testované látky přes buněčnou monovrstvu v obou směrech. Jelikož jsou buňky polarizované a exprimují tak daný transportér jen na jedné straně buněčné membrány, je pohyb substrátů transportérů v jednom směru usnadněn a v druhém limitován. Dle doporučení je pak transportní poměr u substrátů ≥ 2 a je redukován známým inhibitorem daného transportéru.

Pro studium interakcí léčiv s ABC transportéry na orgánové úrovni jsme použili unikátní metodu duálně perfundované potkaní placenty. Naší skupinou bylo již dříve prokázáno, že navzdory mezidruhovým rozdílům ve stavbě potkaní a lidské placenty, je tento model vhodný ke studiu interakcí s ABC transportéry díky podobné lokalizaci, expresi i funkci nejdůležitějších transportérů. Kromě *in vitro* a *in situ* experimentů jsme použili i *ex vivo* akumulační studie provedené na fragmentech či MVM vezikulech z apikální membrány lidského trofoblastu, které nám umožnily studovat transport vybraných léčiv na lidské placentě.

V případě prvního testovaného léčiva TFV jsme *in vitro* nepozorovali interakce s ABCB1, ABCG2 ani ABCC2. Podobně i použití *in situ* perfundované potkaní placenty neprokázalo, že by efluxní transportéry měly vliv na transport TFV. Naměřené hodnoty transplacentární clearance dosahovaly nízkých hodnot v obou směrech, tedy ve feto-maternálním i materno-fetálním, což napovídá tomu, že TFV prostupuje přes placentu jen velmi omezeně. To je v souladu s fyzikálně-chemickými vlastnostmi hydrofilního TFV, u kterého byl navíc zjištěn jen omezený přestup i přes další tělní bariéry jako jsou bariéra střevní a hematoencefalická. Je zajímavé, že klinická data naznačují dostatečnou penetraci tenofoviru do fetálního oběhu, kdy F/M poměr je vyšší než 0,6 a TFV je tak řazen mezi léčiva s vysokým transplacentárním přestupem. Možným vysvětlením může být účast některého jiného transportéru, který je schopen přenést molekulu TFV z mateřského do fetálního kompartmentu.

Proléčivo TDF zvyšuje biodostupnost hydrofilního TFV po perorálním podání; v organismu se během absorpce a následně v cirkulaci poměrně rychle hydrolyzuje na vlastní účinnou látku. Ačkoliv se jedná o látku dostatečně lipofilní, pozorovali jsme, že její přestup přes placentu byl významně omezen činností ABC transportérů, kdy transplacentární clearance byla téměř 12x vyšší ve feto-maternálním směru než ve směru opačném. Proto předpokládáme, že tato molekula je placentárními transportéry

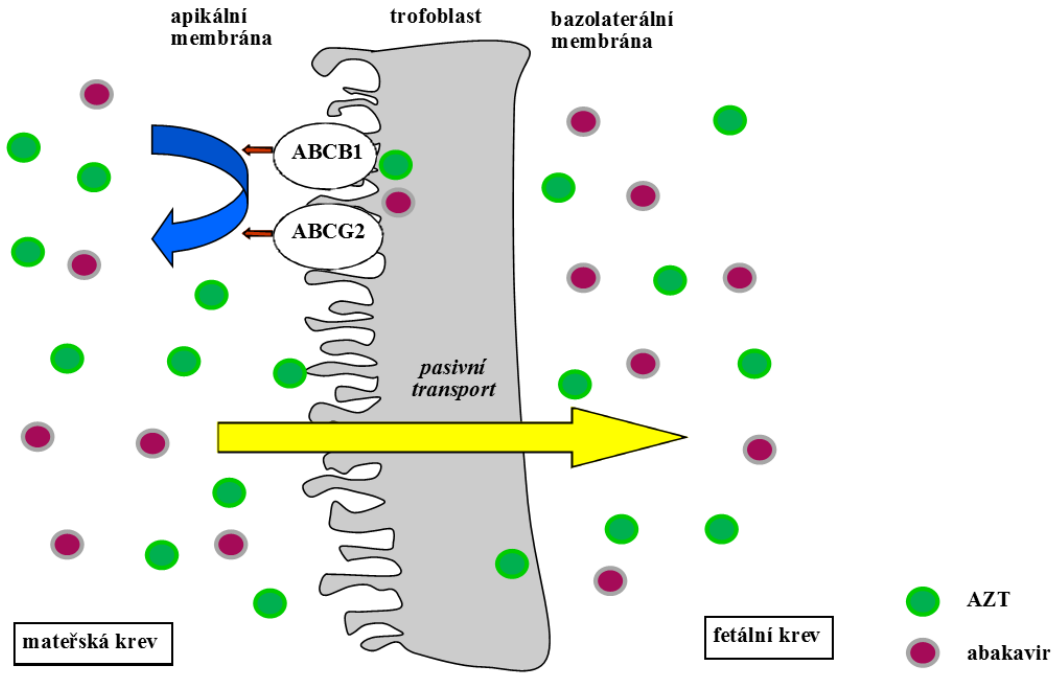
aktivně pumpována zpět do mateřské krve a tím i expozice plodu vůči TDF potažmo TFV bude minimální.



Obrázek 10 Mechanismy transportu TFV a TDF přes placentu. Transplacentární přestup TDF je limitován činností ABCB1 a ABCG2. TFV přechází přes placentu pomocí pasivní difúze a/nebo pomocí dalších transportérů.

V případě léčiv AZT a abakaviru jsme pozorovali velmi podobné charakteristiky transplacentárního přestupu. Zatímco *in vitro* transportní experimenty prokázaly, že obě molekuly jsou substráty ABCB1 a ABCG2, rozdíly v jejich transplacentárních clearance, které by naznačovaly interakce s efluxními transportéry, byly minimální a nesignifikantní. Obě látky tak vykazovaly vysoký transplacentární přestup v obou směrech. Jako důvod tohoto chování předpokládáme vysokou lipofilitu, která může převážit a překrýt případné interakce s transportéry. Za účelem minimalizace efektu pasivní difúze jsme analyzovali transport AZT i abakaviru pomocí experimentálního nastavení využívajícího uzavřený typ perfúzního systému, kdy je na počátku pokusu stejná koncentrace léčiva na obou stranách placentární bariéry. Fetální perfuzát následně recirkuluje a případný pokles koncentrace testované látky v tomto roztoku značí aktivní transport proti koncentračnímu gradientu. U obou látek jsme pak pozorovali

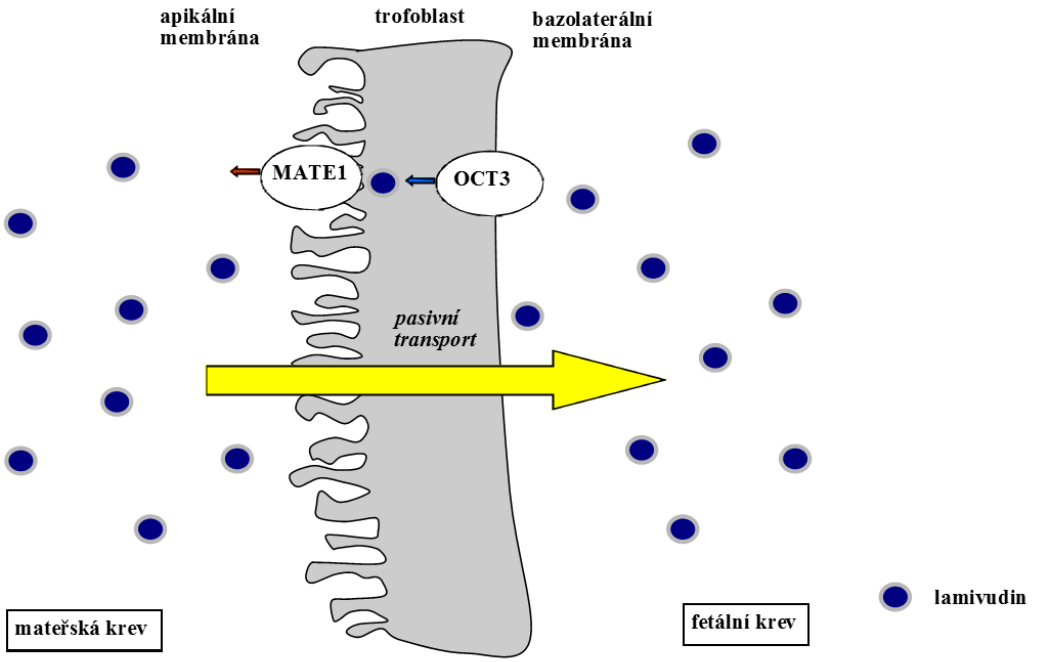
signifikantní pokles v koncentracích léčiv ve fetálním recirkulátu, který byl redukován přidáním specifického ABCB1/ABCG2 inhibitoru. V případě abakaviru jsme testovali jeho interakce s ABC transportéry i přímo v lidské placentární tkáni pomocí *ex vivo* akumulační metody využívající fragmenty lidské placenty. Žádné interakce s ABC transportéry ale nebyly v tomto případě pozorovány. Závěrem lze říci, že AZT a abakavir pravděpodobně přes placentu volně prochází (spekulovat lze i o úloze dalších transportérů, které mohou v tomto přestupu napomáhat), nicméně jejich interakce s ABCB1/ABCG2 by měly být na paměti při sestavování a výběru optimální cART terapie, protože nelze vyloučit, že obě látky mohou ovlivnit transport jiných substrátů těchto transportérů.



Obrázek 11 Mechanismy transportu abakaviru a AZT přes placentu. Obě léčiva navzdory aktivitě ABCB1 a ABCG2, přestupují ve vysoké míře přes placentu do fetální cirkulace pomocí pasivní difúze a/nebo za účasti dalších transportérů.

U lamivudinu jsme na orgánové úrovni neviděli znaky ovlivnění transportu aktivitou ABC transportérů. Transplacentární clearance tohoto léčiva byly v obou směrech srovnatelné a pozorovaný pokles koncentrace ve fetálním recirkulátu nereagoval na přidání známých ABCB1/ABCG2 inhibitorů (data nebyla do finální publikace zařazena). Naše *in vitro* studie rovněž neodhalily, že by lamivudin byl

substrátu na ABCB1, ABCG2 nebo ABCC2. Navíc přidání lamivudinu neovlivnilo transport AZT zprostředkovaný ABCB1 ani ABCG2. Proto nepovažujeme za pravděpodobné, že by tyto transportéry limitovaly transport lamivudinu z matky k plodu či se podílely na lékových interakcích lamivudinu s jinými léčivy. Pozorovali jsme však pH dependentní transplacentární transport lamivudinu zprostředkovaný pravděpodobně transportérem MATE1. Ačkoliv experimenty s MVM vezikuly neprokázaly pH závislost akumulace lamivudinu, studium míry účasti tohoto transportéru na transplacentární farmakokinetice lamivudinu zasluhuje další pozornost.



Obrázek 12 Mechanismy transportu lamivudinu přes placentu. Lamivudin je přenášen přes placentu pomocí pasivních mechanismů. Jeho distribuci k plodu může ovlivňovat zpětný transport zprostředkovaný činností transportérů OCT3 a MATE1.

Dalším cílem této dizertační práce bylo popsat, zda TFV nebo emtricitabin mohou ovlivnit expresi hlavních ABC transportérů. Obě tato léčiva patří mezi nejpoužívanější v terapii HIV infekce a jejich současný bezpečnostní profil nahrává oblíbenosti jejich užívání i u těhotných žen. Jak již bylo zmíněno, u TFV jsme nezjistili potenciál k interakcím s ABC transportéry a naše doposud nepublikovaná data naznačují, že ani emtricitabin není substrát těchto transportérů. Exprese hlavních ABC transportérů byla analyzována ve vybraných orgánech matky i plodu – mozku, střevech,

ledvinách, játrech a také v placentě a to po desetidenní aplikaci testovaných léčiv březím zvířatům. Žádné léčivo nemělo na expresi transportérů vliv, což dále rozšiřuje poznatky týkající se bezpečnosti používání daných léčiv. Zajímavé však je, že po aplikaci TFV byla zjištěna signifikantně vyšší hmotnost placent při zachované hmotnosti plodů při porovnání s kontrolní skupinou zvířat. Tento ukazatel je přitom spojován s rizikem zdravotních komplikací objevujících se v průběhu dospělosti a proto je nutné, aby byl v této oblasti proveden ještě další výzkum pro objasnění dané problematiky.

Závěrem lze říci, že prezentované výsledky přispívají ke komplexnímu obrazu transplacentárního přestupu antiretrovirotik, kde nicméně stále zůstávají oblasti vyžadující další studium.

9 Seznam doposud publikovaných prací kandidátky

9.1 Recenzované publikace v odborných časopisech s IF týkající se tématu práce

Neumanova Z., Cerveny L., Ceckova M., Staud F. *Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta.* AIDS 2014. 28(1): p. 9-17.

$IF_{[2014]}$ – 6.41

Neumanova Z., Cerveny L., Greenwood SL., Ceckova M., Staud F. *Effect of drug efflux transporters on placental transport of antiretroviral agent abacavir*. Reprod Toxicol 2015. **57**:176-82

($IF_{[2015]}$ – 3.38)

Neumanova Z., Cerveny L., Ceckova M., Staud F. *Role of ABCB1, ABCG2, ABCC2 and ABCC5 transporters in placental passage of zidovudine*. Biopharm Drug Dispos 2015.

($IF_{[2015]}$ – 2.34)

Cerveny L., Neumanova Z., Karbanova S., Havlova I., Staud F. *Long-term administration of tenofovir or emtricitabine to pregnant rats; effect on Abcb1a, Abcb1b, and Abcg2 expression in the placenta and in maternal and fetal organs.* Journal of Pharmacy and Pharmacology, 2015

($IF_{[2015]}$ – 2.26)

9.2 Recenzované publikace v odborných časopisech s IF nesouvisející s tématem práce

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($IF_{[2013]}$ – 4.01)

Hofman J., Kucera R., Neumanova Z., Klimes J., Ceckova M., Staud F. *Placental passage of olomoucine II, but not purvalanol A, is affected by p-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2) and multidrug resistance-associated proteins (ABCCs).* Xenobiotica 2015. p. 1-8.

($IF_{[2014]}$ – 2.20)

9.3 Přednášky na konferencích

Neumanova Z., Cerveny L., Staud F. **Study of transport of tenofovir and tenofovir disoproxil fumarate across the rat term placenta.**

3. Postgraduální a 1. Postdoktorandská vědecká konference Farmaceutické fakulty Univerzity Karlovy, 29. – 30. ledna 2013, Hradec Králové, Česká republika

Neumanova Z., Cerveny L., Staud F. **Study of transport of abacavir across the dually perfused rat term placenta**

4. Postgraduální a 2. Postdoktorandská vědecká konference Farmaceutické fakulty Univerzity Karlovy, 28. – 29. ledna 2014, Hradec Králové, Česká republika

Neumanova Z., Cerveny L., Staud F. **Effect of drug efflux transporters ABCB1 and ABCG2 on abacavir transport across the placenta.**

64. Farmakologické dni, 25. – 27. června 2014, Martin, Slovenská republika

Neumanova Z., Cerveny L., Ceckova M., Greenwood SL., Staud F. **Interactions of abacavir with placental nucleoside transporters.**

5. Postgraduální a 3. Postdoktorandská vědecká konference Farmaceutické fakulty Univerzity Karlovy, 3. – 4. února 2015, Hradec Králové, Česká republika

9.4 Postery prezentované na konferencích

Ahmadimoghaddam D., Neumanova Z., Cerveny L., Ceckova M, Staud F. **Transfer of metformin across rat term placenta.**

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Ahmadimoghaddam D., Zemankova L., Neumanova Z., Nachtigal P., Dolezelova E., Cerveny L., Ceckova M., Micuda S., and Staud F. **Expression Profile and Fetus Protective Role of Multidrug and Toxin Extrusion Transporter 1 (Mate1/SLC47A1) and Organic Cation Transporter 3 (Oct3/SLC22A3) at Different Stages of Gestation in Rat.**

62. Farmakologické dni, 25. – 27. června 2012, Košice, Slovenská republika

Neumanova Z., Cerveny L., Staud F. **Study of transport of emtricitabine across the rat term placenta.**

Toxcon, 19. – 21. června 2013, Hradec Králové, Česká republika

Neumanova Z., Cerveny L., Ceckova M, Staud F. **Interactions of tenofovir with drug efflux transporters ABCB1, ABCG2 and ABCC2; role in transport across the placenta.**

BioMedical Transporters, 11. – 15. srpna 2013, St. Moritz, Švýcarsko

Neumanova Z., Cerveny L., Staud F. **Transplacental transport of tenofovir and tenofovir disoproxil fumarate.**

63. Farmakologické dny, 11. – 13. září 2013, Olomouc, Česká republika

Neumanova Z., Cerveny L., Staud F. **Study of transport of tenofovir and tenofovir disoproxil fumarate across the rat term placenta.**

10th International ISSX Meeting, 29. září – 3. října 2013, Toronto, Kanada

Cerveny L., Neumanova Z., Staud F**. Study of transport of antiretroviral drugs zidovudine and lamivudine across the rat term placenta.**

10th International ISSX Meeting, 29. září – 3. října 2013, Toronto, Kanada

Neumanova Z., Cerveny L., Staud F. **Effect of drug efflux transporters ABCB1 and ABCG2 on the passage of abacavir across dually perfused rat term placenta.**

5th FEBS Special Meeting 2014, ABC Proteins: From Multidrug Resistance to Genetic Diseases, 8. – 14. března 2014, Innsbruck, Rakousko

Cerveny L., Neumanova Z., Staud F. **Study of transport of antiretroviral drug zidovudine across the rat term placenta.**

5th FEBS Special Meeting 2014, ABC Proteins: From Multidrug Resistance to Genetic Diseases, 8. – 14. března 2014, Innsbruck, Rakousko

Neumanova Z., Cerveny L., Staud F. **Interactions of abacavir with placental drug efflux transporters ABCB1 and ABCG2.**

Solvo Meet the experts, Transporter conference 2014, 1. – 4. dubna 2014, Budapešť, Maďarsko

Ceckova M., Neumanova Z., Reznicek J., Cerveny L., Staud F. **Translacental pharmacokinetics of lamivudine is not affected by ABC drug efflux transporters.**

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Neumanova Z., Cerveny L., Staud F. **Effect of drug efflux transporters ABCB1 and ABCG2 on abacavir transport across the placenta.**

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Neumanova Z., Cerveny L., Ceckova M, Jiraskova L., Staud F. **Úloha nukleosidových transportérů v transplacentárním přestupu abakaviru.**

XV. Mezioborové setkání mladých biologů, biochemiků a chemiků, 12. – 15. května 2015, Milovy, Česká republika

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Cerveny L., Neumanova Z., Karbanova S., Havlova I., Staud F. **Long-term administration of tenofovir or emtricitabine to pregnant rats; effect on Abcb1a, Abcb1b and Abcg2 expression in maternal and fetal organs.**

13th European ISSX Meeting, 22. – 25. června 2015, Glasgow, Velká Británie

Neumanova Z., Cerveny L., Karbanova S., Jiraskova L., Greenwood SL., Glazier J., Ceckova M., Staud F. **Role of equilibrative nucleoside transporter 1 (ENT1) in transfer of abacavir across the placenta.**

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Karbanova S., Neumanova Z., Cerveny L., Staud F. **Nucleoside transporters does not affect transport of zidovudine across the placenta.**

65. Farmakologické dny, 16. – 18. září 2015, Olomouc, Česká republika

10 Ocenění

Za posterovou prezentaci s názvem „**Effect of drug efflux transporters ABCB1 and ABCG2 on the passage of abacavir across dually perfused rat term placenta“** byla udělena cena „**ABC2014 Student Fellowship**“

autoři: **Neumanova Z.**, Cerveny L., Staud F.

5th FEBS Special Meeting 2014, ABC Proteins: From Multidrug Resistance to Genetic Diseases, 8. – 14. března 2014, Innsbruck, Rakousko

Za posterovou prezentaci s názvem „**Effect of drug efflux transporters ABCB1 and ABCG2 on abacavir transport across the placenta“** byla udělena cena „**Najlepší poster v posterovej sekcii doktorandov**“

autoři: **Neumanova Z.**, Cerveny L., Staud F.

64. Farmakologické dni, 25. – 27. června 2014, Martin, Slovenská republika

Za publikaci s názvem „**Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta“** byla udělena cena „**Nejlepší práce v kategorii experimentální farmakologie České společnosti pro experimentální a klinickou farmakologii a toxikologii České lékařské společnosti J. E. Purkyně za rok 2014**“

autoři: **Neumanova Z.**, Cerveny L., Ceckova M., Staud F.